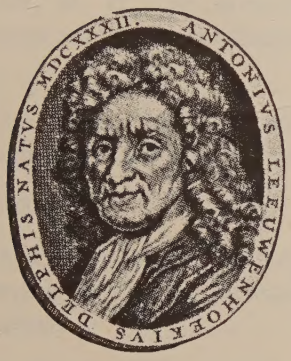
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SWETS EN ZEITLINGER
BOEKHANDEL EN UITGEVERSMAATSCHAPPIJ — AMSTERDAM

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STUDIES OF BACTERIA IN DANISH FRUIT-WINES

by

ERIK OLSEN

(Received March 1, 1948).

During recent years, in which the Danish manufacturers of fruit-wines have been forced to use an inferior, unrefined 60 % beet-sugar syrup, this industry has been greatly troubled by a ,,disease" in sweet wines rich in alcohol.

This disease may occur already in the course of fermentation, at first decreasing, later on increasing the acidity of the wine, but most often it only occurs in later stages, especially when it befalls the rich Vermouth types and cherry-wines either with or without addition of apple-juice. The disease may appear in wines of 19 per cent by volume of alcohol. Initially the wine becomes turbid and a strong development of carbon dioxide takes place, which can force the corks out of the bottles. The disease appears with equal frequency in the casks. By and by the wine becomes very turbid and the colour changes, for instance the red cherry-wine turns brownish. Already in the very first stage of the disease a characteristic, unpleasant acid odour is produced, which increases and becomes more acetic. A brownish, amorphous, voluminous precipitate develops consisting of precipitated dye-stuff and containing numerous long, entangled threads and gram-positive rods, single or in chains. The size of these rods is 0.75×2—$10\ \mu$. Sometimes mannitol is found.

In normal fruit-wine the acidity is 3.0—3.5 g per l calculated as tartaric acid, with 0.1—0.35 g volatile acid (calculated as tartaric acid) and pH is 3.5—3.8. But after the beginning of this disease the acidity may increase and reach 6—9 g per l, 7.5—10 % of which is acetic acid. This can be tasted already when the acidity is 4—5 g per l.

The disease appears at temperatures from 10—38° C. and takes

in general 3—6 months to develop. At higher temperatures, however, it develops more rapidly. This is, therefore, a typical „tourne-pousse" disease. The French word „tourne" (German: umschlagen, English: lactic souring) indicates a disease, which manifests itself by turbidity, acid-formation, unpleasant bitter taste, often so-called „Maüseln" (mousy taste), the colour at the same time changing from red to brown, and which gives a precipitate especially after access of air. „Pousse" indicates the development of carbon dioxide caused by the same bacteria which form the acid.

In a few cases a similar disease has been found in wines with a lower percentage of alcohol, but a high percentage of nitrogen. This high percentage of nitrogen was due to a very inferior sugar-syrup. These samples of wine were completely spoiled before bottling, were very turbid and had a most unpleasant acid taste. A microscopical examination showed numerous rods varying in length and thickness, but no filaments. Although this case is rare in Danish fruit-wines, it is of interest to study the active bacteria on account of their great activity in the wine, all the more so as no detailed investigation of bacteria in Danish fruit-wines exists.

Finally the results of a study of a fruit-wine, which became very ropy during the main fermentation, will be presented.

I. Earlier Descriptions of the Bacteria causing Diseases in Wine.

Practically all the bacteria causing diseases in wine already described in the literature can be classified among the rod-shaped, heterofermentative lactic acid bacteria. These bacteria, which next to inactive lactic acid (sometimes with a surplus of laevolactic acid) form acetic acid, CO_2 and some ethanol, and from laevulose in general mannitol, have been collected by Orla-Jensen (27) in the genus *Betabacterium* in which he puts up 3 species:

1) *Bbm. breve* characterized by fermentation of arabinose and xylose, optimum temperature about 30° C., size 0.7 × 2—4 μ. On account of the arabinose fermentation Frank (9) has proposed the name *Bbm. arabinosaceum*, which name was later accepted by Orla-Jensen (28).

2) *Bbm. longum* does not ferment arabinose, but sometimes xylose. Optimum temperature about 40° C. Size varying from short rods to filaments.

3) *Bbm. caucasicum* from kephir grains is difficult to cultivate. It chiefly ferments arabinose and when freshly isolated also lae-

vulose, glucose and saccharose. Optimum temperature above 30° C. Shorter and longer rods.

The Betabacteria in general have a rather low pH optimum, at about 5.3 (30).

PEDERSON (33) and BERGEY's Manual of Determinative Bacteriology (5th ed. 1939) divide this group in a similar way, but put up some more species, chiefly based upon the cardinal points of the temperature curve and fermentation of arabinose, saccharose, raffinose and lactose. The arabinose-fermenting species are divided in *Lactobacillus brevis* (a synonym of *Bbm. arabinosaceum* Orla-Jensen), *pastorianus* and *buchneri*. The latter has slightly higher cardinal temperatures than *Lbc. brevis*, but the difference seems — according to PEDERSON's original work — to be so insignificant that a classification based hereupon can be difficult. *Lbc. pastorianus* does not ferment xylose and seems more likely to form longer cells (2—20 μ). By reason of priority *Bbm. longum* Orla-Jensen is called *Lbc. fermenti* Beijerinck.

In some cases the bacteria described — especially when they are originary from ropy wine — probably belong to the spherical, heterofermentative lactic acid bacteria, *Betacoccus* Orla-Jensen (*Leuconostoc* van Tieghem), and in a very few cases the isolated bacteria have been rod-shaped, homofermentative lactic acid bacteria belonging to the species *Streptobacterium plantarum* Orla-Jensen.

As to the diseases in wine caused by these bacteria, already PASTEUR (32) distinguished between bitter wine (ferment de l'amèr), lactic soured wine (ferment de la pousse) and ropy wine (ferment de la graisse) and he noted the formation of mannitol in ropy wine.

GAYON and DUBOURG (11) isolated „le ferment mannitique" from Algerian lactic soured wine and showed that besides inactive lactic acid, acetic acid, CO_2 and some succinic acid and glycerol it formed mannitol from laevulose. From glucose, moreover, some ethanol was formed, but no mannitol. In 1901 (12) they found that the higher the initial degree of acidity was in wine, the less mannitol it formed. This bacterium fermented xylose, but not arabinose, and had opt. temp. at 35° C. It is most certainly identical with *Bbm. longum* Orla-Jensen.

EKREM-RESIT (6) noted this bacterium to develop a greater amount of alcohol and lactic acid from glucose in yeast water + $CaCO_3$ than in an acid medium and next to this glycerol. From laevulose 61 % was converted into mannitol in the presence of $CaCO_3$, but only 9.1 % in its absence. In the former case moreover quite a large amount of glycerol is formed along with some ethanol. Similar bacteria were isolated by MAZÉ and PERRIER (20) and by MAZÉ and PACOTTET (21).

Slimeforming bacteria, often occurring in the white wines (more rarely in the completely fermented red wines), which make the wines turn viscous and slimy, induce an insipid taste and cause gas

formation, were described by KAYSER and MANCEAU (17). These were short ovoid organisms in short chains surrounded by slime. In the course of time the cells became longer and the chains shorter. The optimum temperature was 25—30° C.

These organisms were apparently either Betabacteria or Betacocci. The study of ropy wine reported on in this paper undoubtedly reveals this organism as a *Betacoccus*. Betabacteria seldom form slime in contradistinction with Betacocci, which generally form dextran from saccharose. Yet, MAYER (19) and PERQUIN (34) have described a vigorously slimeforming *Bbm. vermiforme* from ginger-beer plant, tibi grains etc. It ferments arabinose and xylose vigorously and corresponds best with the characteristics of a slimeforming *Bbm. arabinosaceum.*

MÜLLER-THURGAU and OSTERWALDER (22) separated lactic souring from formation of mannitol even if these are caused by the same bacteria, as the formation of mannitol only occurs, when there is laevulose left. They consider the initial decomposition of acid as beneficial to wines of great acidity, but later lactic acid may be produced by the same bacteria, thus spoiling the wine. In this work and in one of later date (23) they described *Bact. mannitopoeum, gracile, intermedium* and *gayoni* (GAYON and DUBOURG's „ferment mannitique").

Bact. mannitopoeum, which was chiefly isolated from fruit-wines with lactic souring, is, according to the description, identical with *Bbm. arabinosaceum* Orla-Jensen (*Lbc. buchneri* Bergey), as it fermented arabinose and xylose.

Bact. gracile formed very short, almost coccoid cells, in pairs or in chains, and as mentioned in BERGEY's Manual, it is possible that it has been a *Betacoccus.* It could stand but a comparatively low concentration of alcohol and had an opt. temp. at 22—26° C. It was less apt to give lactic souring than *Bact. mannitopoeum,* but decomposed malic acid vigorously.

Bact. intermedium from red wine fermented xylose, but not arabinose and decomposed malic acid vigorously. This bacterium, as well as *Bact. gayoni,* which did not decompose malic acid, is probably identical with *Bbm. longum* Orla-Jensen.

While the above bacteria have been found in wine of a relatively low alcohol percentage and can only stand 12—15 % by volume of alcohol, later quite a number of bacteria spoiling fortified wines of 16—20 % alcohol have been described.

Thus FEVRIER (8) found a bacterium in South African wines of 19—20 % by vol. of alcohol. It formed much volatile acid and made the wine turn darker. It could stand a large amount of sulphur dioxide and was very heat-resistant (5 minutes up to 80° C., 3 days in succession). Temperature optimum was rather high.

NIEHAUS (24) found a similar, filiform bacterium in fortified South African wine. It grew at 18 % by vol. of alcohol and formed mannitol. A growth factor from yeast was necessary.

In port-wine of 18.1 % of alcohol D'ESTIVAUX (7) found a long, rod-shaped bacterium forming volatile acids.

Spoilage of Californian sweet wines has been examined by DOUGLAS and MCCLUNG (4). This disease revealed itself by the formation of a voluminous, flocculant precipitate, while the wine itself was absolutely clear. The chemical change was only small, yet, some lactic and acetic acid was formed and sometimes gas developed. The change in taste was insignificant. In the precipitate a lot of long, entangled filaments resembling a bunch of wet hair, were found, for which reason the authors termed them „hairbacillus". This bacterium was hard to cultivate and growth only resulted in sweet wine diluted with equal parts of water, whilst only in one case colonies were obtained by spreading on agar of the same composition. Optimum pH was 4.1—4.3 and optimum temperature 20-25° C., while hardly any growth was found at higher temperatures. The growth was inhibited by 60 mg SO_2/l or a total acidity of 0.5 g/100 ml. The alcohol limit was about 22 % and the heat resistance slight. From the incomplete descriptions of this bacterium it may be termed a *Betabacterium*, but the species has to be left open.

Lactic acid bacteria which can stand 22-26 % of alcohol, have been found in Japanese Saké by OTANI (31).

DOUGLAS and CRUESS (3) have described a tourne-disease from not completely fermented and not fortified Californian wines. The disease occurred after the fermentation or in springtime, when the temperature increases, as „silky haze", and with a vigorous increase of acidity, sometimes the so-called „Maüseln". The bacterium causing this disease could only be cultivated in diluted wine or grape juice. From laevulose it formed lactic acid and acetic acid, but no mannitol or CO_2. It grew at pH 3.0-7.6 with optimum temperature between 31-37° C., poor growth at 40-42° C. and none at 50° C. It was killed in grape juice by heating up to 57° C. for 10 minutes and was inhibited by 75 mg SO_2/l. In a medium with 16.2 % by vol. of alcohol it developed in one month and in one with 18 % in 2 months. The authors called this bacterium *Lactobacillus hilgardii*. DOUGLAS and VAUGHN (according to CRUESS (2)) are of the opinion that it is closely related to *Lactobacillus plantarum* (*Streptobacterium plantarum* Orla-Jensen).

ORLA-JENSEN also has found *Sbm. plantarum* in Danish cider of 5-8 % by vol. of alcohol, but this group of lactic acid bacteria seems to occur much more rarely in wine than Betabacteria.

This genus is wide-spread in nature and numerous species have been described more or less exactly, thus *Lbc. fermentum* Beijerinck (SMIT (40)) identical with *Bbm. longum* Orla-Jensen. SMIT points out in this paper that „nach der jetzt angenommenen Nomenklatur der sporenlosen Organismen wäre *Lactobacterium* richtiger" than *Lactobacillus* chosen by BEIJERINCK (1), a point of view constantly maintained by ORLA-JENSEN. In view of priority the latter name is retained in the American nomenclature.

HENNEBERG (14) has isolated and described a series of bacteria to be classified among the Betabacteria, *i.e. Bact. brassicae fermentatae* from Sauerkraut, *Bact. hayducki* and *buchneri* from pressed yeast, *Saccharobacillus pastorianus* from lactic soured Belgian beer. Most of these bacteria have optimum temperatures at 34-38° C., are arabinose-fermenting and are, as far as can be said, identical with *Bbm. arabinosaceum.*

From leaven KNUDSEN (18) has isolated 3 types of Betabacteria — α, β and γ — of which α was identical with *Bbm. arabinosaceum*, and γ to *Bbm. longum* if anything, but difficult to cultivate. β was slightly atypical, as the arabinose fermentation was fluctuating. It fermented mannose vigorously.

In a series of papers PETERSON and co-workers (35, 36, 37, 38) have described *Lbc. pentoaceticus*, isolated from corn silage. It was identical with *Bbm. arabinosaceum*, as it fermented arabinose and xylose. It formed mannitol from laevulose and was able to ferment mannitol to acetic acid, formic acid, lactic acid and ethanol. Some of the strains of *Bbm. arabinosaceum* described by ORLA-JENSEN (28) also ferment mannitol.

In a later paper STILES and collaborators (41) described a series of mannitol forming bacteria isolated from fermenting cereal infusions. On the basis of their fermentation products and fermentation of sugars they were identified with *Bact. mannitopoeum* Müller-Thurgau et Osterwalder, *Bact. gayoni* Müller-Thurgau et Osterwalder, *Bbm. arabinosaceum* Orla-Jensen (strain 11, differing from the usual in that it hardly ferments xylose), and finally *Lbc. fermentum* Beijerinck.

It has not been tested, whether also these bacteria were able to develop in acid, alcoholic media, but they are most likely to, as it will be proved in this paper that Beta- and Streptobacteria from silage are able to develop at rather high alcohol concentrations. Besides, FRANK (9) has also found that many Betabacteria, especially from Emmenthal cheese and leaven, develop rapidly in beerwort with an alcohol content of 10 %.

II. The Author's own Investigations.

A description of the disease occurring in the samples of wine examined has already been given in the introduction. In the following the mode of isolation of the harmful bacteria and investigations of some of their properties will be reported on.

1. Isolation of bacteria.

By direct microscopical examination of wine with a low alcohol content, but very rich in nitrogen, produced by the addition of a very unrefined beet-sugar syrup (wine A), thick and thin, short

and long rods, single or in chains, were observed. By spreading of several of these samples of wine on casein-peptone-yeastautolysate-agar (C-Y-agar) all of them appeared to give rise to two types of colonies:

A1: rather large (about 2 mm in diam.), white, smooth colonies of slender rods, single or in chains of 3—4.

A2: small (0.25—0.5 mm in diam.), white, smooth colonies of short, thick, partly coccoid rods, single or in short chains.

These bacteria were as easy to cultivate as common lactic acid bacteria from other sources.

On the contrary it appeared very difficult to isolate the bacteria, which during recent years have spoilt so much Danish fruit-wine of the fortified type of 16—20 % by vol. of alcohol. By direct microscopical examination of the heavy, brown, amorphous precipitate in such a wine showing vigorous gas development and acid formation, numerous slender rods, single or up to 3—4 together, 0.75 × 2—10 μ, as well as many threads and long chains, were observed.

Spreading was tried on many different media, both on plates and in Burri tubes, but no growth of these bacteria took place. In liquid media of different pH and composition, such as C-Y-bouillon and media containing yeast-water, yeast-autolysate, extract of maltsprouts, beet-sugar syrup, inverted saccharose, laevulose, glucose, malic, tartaric, or citric acid inoculation with the wine samples induced but very rarely a feeble growth. Generally it lasted very long before they grew in germfree filtered cherry-wine, and agar media prepared with wine gave no growth either. Varying the temperature between 20—37° C. had no influence.

However, experiments with a medium adjusted to various pH and either with or without the addition of 10 % by vol. of alcohol furnished the reason for the poor results obtained thus far. The medium used was:

10 % yeast-water	40 %
yeast-autolysate, 1 % N	10 %
5 % maltsprouts extract	20 %
glucose + laevulose	2 %
tap water	30 %

This medium designed as YYM-medium after autoclaving was adjusted to pH 5.8, 5.0, 4.2, 3.6, whereupon 10 % by vol. of alcohol

(germfree filtered) was added to half of the batch. After having been distributed in sterile tubes with screw caps to prevent evaporation of the alcohol, inoculation from different samples of wine took place. An incubation at 30° C. for a month in the media without alcohol had allowed no or merely very slight growth, whereas the presence of alcohol in the tubes had promoted the growth vigorously, especially at pH 4.2 and 3.6.

Thus apparently alcohol and acid reaction are necessary for the cultivating of these bacteria, which never grow in raw fruit-juice. Now a spreading in C-Y-agar in Burri tubes, the pH of which, after melting and cooling down to 45° C., was brought down to 4.5 by a mixture of equal parts of malic, tartaric and citric acid and which were provided with 10 % germfree filtered alcohol, was attempted. After inoculation by the diluted bacterial suspensions the agar was rapidly cooled down to keep the alcohol from evaporation and the tubes were closed with a paraffin cap. After a fortnight at 30° C. well developed colonies had appeared and in the denser suspensions the agar had been split by formation of gas.

Spreading was made from a Vermouth type with about 18 % by vol. of alcohol and from several samples of cherry-wine and various types of port-wine, mostly produced from apples with the addition of a 60 % unrefined beet-sugar syrup. The Vermouth type originated from another factory than the other samples. By spreading from the Vermouth type, 2 types of colonies appeared:

V1: small, white colonies with fimbriate edges of rather short, gram-positive, micro-aerophilic rods in chains of 5—8 cells.

V2: larger (2 mm in diam.), white colonies, with edges only slightly fringed, of somewhat longer gram-positive, anaerobic rods, in chains of 5—8 cells.

From cherry-wine and port wine 2 types of colonies likewise appeared:

B1: rather large (2 mm in diam.), white, smooth, lens-shaped colonies of rods very much like V2. A lot of very long chains and „balls" of thread, however, were noticed. They were anaerobic.

B2: very small, white, angular colonies of rods and filaments, just like B1 and also anaerobic.

Both the B and V2 bacteria gradually grew microaerophilic.

These colonies were inoculated in YYM-medium with 10 %

inverted sugar syrup and adjusted to pH 4.5 and 3.5 by means of malic acid; 12 % by vol. of alcohol being added. Already after 20 hours at 30° C. gas developed in most of the tubes adjusted to pH3.5. After another fortnight they had developed equally at pH3.5 and 4.5 with the exception that at pH 3.5 the cells generally were longer.

The following state of development was noted:

V1: slightly turbid. The precipitate consisted at the bottom of a white rather loosely connected layer of bacteria, and on top of a fluffy, brownish precipitate from the medium. The cells were 0.75 × 2—5 μ. No chains or threads at pH 4.5, but some at 3.5.

V2: similar to V1, but a few threads were noticed at pH 4.5 and some more at 3.5.

Apparently there is no difference between V1 and V2.

B1: the medium clear. Precipitate as in V, but with longer, slender, chain-thread-forming rods, 0.75 × 3—12 μ. In some tubes both at pH 3.5 and 4.5 a big, cauliflower-like, somewhat slimy coherent colony appeared, consisting of long entangled, chains and filaments.

B2: Not before 3 weeks at 30° C. a feeble growth appeared. The medium was clear with only a slight precipitate. The size of the cells was 1 × 1—10 μ, single and in short chains.

Thus the B2 strains grow somewhat more slowly than the others. The appearance of the big kephir-grainlike colonies of bacteria is quite interesting. MÜLLER-THURGAU and OSTERWALDER (22) have described similar zoogloea in *Bact. mannitopoeum, gracile* and *gayoni*. ORLA-JENSEN also mentions such, but smaller aggregates in *Bbm. caucasicum.*

Next to these lactic acid bacteria some spore-forming bacteria, chiefly *Bac. mesentericus*, were noticed in wine V, but as these bacteria could not grow at pH lower than 5.7, they are scarcely of any importance in wine. Most likely they originated in spore-form from the sugar-syrup, as similar bacteria were isolated therefrom.

Some authors of earlier date have mentioned the isolation of sporeforming bacteria from bitter or lactic soured wine (see f.i. MÜLLER-THURGAU and OSTERWALDER (22)), but in most cases the faults are probably due to the lactic acid bacteria, a pure culture of which is harder to obtain.

2. The properties of the isolated bacteria.

A series of titrations and pH measurements made during the fermentation may illustrate the attacks on wine by B- and V-bacteria. The cider was prepared from apples with the addition of raisins and brown beet-sugar syrup. The acidity is calculated in grams of tartaric acid per l.

TABLE I.

Time of fermentation (in days)	1	2	3	4	7	9	10	14
Acidity	5.00	4.98	4.96	4.88	4.00	3.20	3.15	3.10
pH	3.58	3.59	3.60	3.66	3.80	3.94	3.95	3.95

It happens sometimes that the yeast reduces the acidity during the fermentation by its decomposition of malic and citric acid, but when pure cultures are used the amounts are as a rule quite small. A reduction of 38 % of the original acidity can by no means be produced by yeast. Therefore, undoubtedly, the bacteria present will have decomposed the malic acid, a feature which could also be noted in pure cultures of these bacteria.

During the secondary fermentation and the storage a vigorous acid formation and gas development occur. Thus, 7.6 g acid per l calculated as tartaric acid was found in a bottled wine, which had made the cork fly. Originally the acidity of this wine was 3.5 g per l, while the pH was almost unchanged, $\pm$ 3.6. Such a spoiling of wine with 16—20 % by vol. of alcohol is a quite common phenomenon.

Sometimes mannitol is found, but not always and even the isolated bacteria cannot always form this alcohol from laevulose. This most probably depends on the fact, whether the remaining sugar is laevulose, glucose or saccharose. Mannitol is not formed from glucose and but rarely from saccharose. The literature furnishes but few examples of bacteria able to form mannitol from saccharose without previous inversion, *i.e. Bact. mannitopoeum* and STILES, PETERSON and FRED's species 19 and 36 (41) which, however, formed merely very small quantities.

All the isolated bacteria had the properties common to the lactic acid bacteria.

The products formed by the fermentation of glucose and laevulose were identified in the YYM-medium with 2 % sugar without calcium carbonate. The media inoculated with B- and V-bacteria were provided with 10 % alcohol. As it appeared impossible completely to avoid evaporation of alcohol from the flasks the results of the estimations of alcohol were not identical for the various cultures. In the glucose media its amount was left unchanged with B1 and V1 but had slightly increased with B2. In the laevulose media it was unchanged with V1, but it had increased rather strongly with B1 and B2.

The production of CO_2 by the A-bacteria has not been estimated. The CO_2 produced by the B- and V-bacteria was absorbed by carbonate free NaOH ($\pm$ 18 n) in a test-tube connected with the culture flask by a glass tube. After the fermentation this test-tube with its contents was inserted into the fermentation flask and the total content of CO_2 was estimated on an aliquot of the medium. After acidification by means of sulphuric acid the CO_2 was driven over into barium hydroxide by a CO_2-free current of air. As some of the volatile acids pass over along with the CO_2, it is necessary to filter the barium carbonate, wash it with a saturated solution of barium chloride, dissolve with a known quantity of n/10 hydrochloric acid and titrate the excess. Control analyses made in this way have shown good agreement, whereas, when the volatile acids are not taken into account, a surplus of 5-10 % CO_2 will be found, depending on the quantity of these acids and the duration of aeration.

Lactic acid was estimated in the residue left after the distillation of the volatile acids. By means of chromic-sulphuric acid it was oxidised to acetic acid, which was distilled off and titrated according to Szeberényi (42). Ethanol was likewise estimated by oxydation with chromic-sulphuric acid to acetic acid and titrated as such. Mannitol was not estimated quantitatively, but was identified in the residue left after evaporation of an alcoholic extract of the dried medium by the characteristic white rosettes of needle shaped crystals.

In Table II the results of the analyses are presented, the amounts of the different components indicated in % of fermented sugar. Acetic acid was practically the only volatile acid.

The results of the fermentation show that the bacterium-type A1 is homofermentative and that all the others are heterofermentative lactic acid bacteria, which form mannitol from laevulose.

The bacteria A1 and A2, easy to cultivate, were tested as to their ability of fermentation of the „sugar-series" in C-Y-bouillon at pH 6.7 provided with 2 % of the sugar dealt with. For the B- and V-bacteria the YYM-medium was used, which after autoclaving at pH 6.8 was adjusted to pH 4.5 by means of sulphuric

TABLE II.

Fermentation products by fermentation of glucose and laevulose in % of fermented sugar.

Bacterium		Sugar	Lactic Acid		Acetic acid	Ethanol	CO_2	Mannitol
Type	Strain		Sort	Amount				
A1	13.1	Glucose	i + d	43.2	3.8	0.6	—	—
A1	13.1	Laevulose	i	54.5	5.9	1.4	—	—
A1	26.1	Glucose	i + d	53.4	2.9	0.7	—	—
A2	13.2	Glucose	i	26.7	16.9	1.8	+	—
A2	13.3	Glucose	i	47.4	9.4	1.1	+	—
A2	13.3	Laevulose	i	27.0	12.1	4.4	+	+
B1	1	Glucose	i	48.2	15.3	—	11.8	—
B1	1	Laevulose	i	25.7	11.6	—	13.2	+
B2	2a	Glucose	i	48.9	16.2	—	22.4	—
B2	2a	Laevulose	i	25.5	9.4	—	12.6	+
V1	a	Glucose	i	47.5	9.8	—	16.7	—
V1	a	Laevulose	i	25.8	8.2	—	14.7	+

acid and having been provided with 10 % ethanol was measured in sterile test-tubes. After inoculation the tubes were corked. After the A-bacteria had been kept for 2 weeks and all the others for 4 weeks at 30° C. the acidity was estimated.

For the detection of the decomposition of malic acid use was made of a sugar-free medium provided with 1 % of malic acid and adjusted to pH 4.5. After a lapse of generally three weeks the medium was titrated with 0.1 n NaOH. A decrease of the acidity proves conversion of the malic acid into lactic acid and CO_2, *i.e.* a reduction of the number of carboxyl groups. Further estimations have been made in flasks containing 150 ml medium, the CO_2, lactic acid and acetic acid formed in these were estimated. In fact some acetic acid is also formed in the course of the process. In Table III the results of the quantitative estimations are taken up, the figures indicating the amounts of products in % of decomposed malic acid. The latter had been estimated by titration before supply to the medium and its complete decomposition is taken for granted.

TABLE III.

Decomposition of malic acid.

Bacterium		Lactic acid	CO_2	Acetic acid
Type	Strain			
B1	1	71.4	20.6	4.3
B2	2a	77.1	18.0	3.1
V1	a	67.6	20.5	7.0

In Table IV the results of the fermentation of the „sugar series" are shown, indicated in pro mille lactic acid, and in the last column decomposition and non-decomposition of the malic acid are indicated by + and — respectively.

TABLE IV.

.cterium		Glycerol	Xylose	Arabinose	Sorbitol	Mannitol	Laevulose	Glucose	Mannose	Saccharose	Raffinose	Inulin	Salicin	Milk	Laevulose + Glucose	Saccharose, inverted	3% beet-molasses, inverted	Malic acid
;pe	Strain																	
1	13.1	0.0	2.0	0.0	0.0	4.5	10.5	9.5	10.5	11.5	0.0	11.0	7.5	12.6				+
1	26.1	0.5	2.0	0.0	0.0	4.5	10.5	10.0	10.0	10.5	0.0	10.0	7.5	12.4				+
2	13.2	0.0	11.0	9.0	0.0	0.0	5.0	5.0	0.5	4.5	2.5	0.0	0.0	1.8				—
2	13.3	0.0	10.5	8.0	0.0	0.0	5.0	5.0	0.0	0.0	0.0	0.0	0.0	2.0				—
2	26.2	0.0	11.5	8.0	0.0	0.0	5.0	5.0	0.0	0.0	0.0	0.0	0.0	1.1				—
A2	26.5	2.0	11.5	8.0	0.0	0.0	5.0	4.5	1.3	0.5	0.5	0.0	0.0	2.0				—
B1	1	0.0	0.0	0.0	0.0	0.0	3.0	1.0	0.5	0.0	0.0	0.0	1.0	0.0	3.0	3.0	2.5	+
B2	2a	0.0	0.5	0.0	0.0	0.0	4.5	0.0	0.0	1.0	0.0	0.0	0.0	0.0	4.0	4.0	1.5	+
B2	2b	0.0	0.5	0.0	0.0	0.0	5.5	0.5	0.5	0.0	0.0	0.0	0.5	0.0	3.5	3.5	1.5	+
V1	a	0.0	0.5	0.0	0.0	0.0	5.0	3.5	0.5	2.0	0.5	0.5	1.0	0.0	4.0	3.0	2.5	+
V2	b	0.0	0.5	0.0	0.6	0.0	1.0	1.5	0.5	0.0	0.0	0.0	0.0	0.0	2.0	0.0	2.0	+

The A1 strains 13.1 and 26.1 are absolutely identical. It is a homofermentative lactic acid bacterium with optimum temperature at 30° C. It is most probably to be identified with *Sbm. casei*, due to the lack of fermentation of arabinose, melibiose and melizitose

and the growth in milk (which, however, with the addition of calcium carbonate is only weakly proteolysed). *Sbm. plantarum* generally ferments melibiose and arabinose and grows poorly in milk.

The A2 strains are typical *Bbm. arabinosaceum* and undoubtedly these bacteria are identical with *Bact. mannitopoeum*. The B strains are characterized by the fact that they practically only ferment laevulose and inverted saccharose. As substances, promoting the growth of these bacteria like the unrefined sugar does, might be expected to occur in molasses, inverted molasses have also been added, but it appeared to be fermented less vigorously than inverted saccharose.

The V-strains are slightly different. Besides fermenting laevulose and glucose, V1 also weakly ferments saccharose and salicin. V2 is on the whole more feeble and does not ferment either saccharose or salicin. Both B- and V-strains ferment xylose and mannose weakly.

These bacteria are supposedly identical with or closely related to KNUDSEN's γ-strains (18) from leaven, these being Betabacteria, which do not ferment pentoses and most often very few hexoses. KNUDSEN states as characteristic for these strains, that they are more anaerobic than the other types and difficult to cultivate on artificial media. His cultures thrive best in unfiltered cereal mash provided with yeast-autolysate. Beerwort was much inferior. The above V-strains were initially anaerobic and besides ask for a very specific nutrition. For these also wort is a poor medium and yeast-water is much better.

Morphologically there is good agreement too, as the γ-bacteria mostly are rather long, varying in length from 3 μ to long threads. These bacteria correspond nearest with ORLA-JENSEN's *Bbm. longum*, to which species probably also *Bact. intermedium* and *gayoni* MÜLLER-THURGAU et OSTERWALDER belong.

The growth of the isolated bacteria at different alcohol concentrations and different pH has been tested in YYM-medium, provided with 5 % inverted very impure 60 % beet-sugar syrup. The medium was adjusted to pH 4.3 with equal parts of malic, tartaric and citric acid. In the alcohol series the calculated amounts of germfree filtered 96 % alcohol were added after autoclaving. The pH serie was provided with 10 % by vol. of alcohol and the pH adjusted with sulphuric acid, resp. NaOH and controlled by a glass

electrode. 10 ml of the media were measured in tubes with screw-caps. The growth was judged by the degree of turbidity of the medium, as well as by titration after 12 days at 30° C. The optimum conditions are marked quite distinctly by the titration results gathered in Tables V and VI. The figures indicate pro mille acid calculated as lactic acid, of course after subtraction of control tubes.

TABLE V.

The Influence of the Alcohol Concentration.

Alcohol % by vol.	Type	A1	A2	B1	B1	B2	V1
	Strain	13.1	13.2	1	2	2a	a
0		10.6	6.5	0.1	0.2	0.1	2.5
1		10.4	5.6	0.7	0.7	0.5	2.9
5		7.9	3.8	2.5	2.7	2.7	2.9
10		7.0	2.5	2.7	2.7	2.7	2.5
15		1.1	1.6	2.3	2.7	2.3	2.5
17.5		0.5	0.9	2.3	2.0	2.3	2.0
19		0	0	0.2	0.2	0.2	0.1
20		0	0	0	0.1	0	0.1
21		0	0	0	0	0	0.1

With concentrations of alcohol above 20 % approximately, practically no formation of acid took place in the course of 14 days. Up to 23 % of alcohol, however, the media were slightly turbid and cells could be detected microscopically, so probably some acid formation would also have occurred here after a longer period. Besides, the cells had grown longer and more chain- and thread-forming with increasing alcohol concentration, a feature very common in lactic acid bacteria, when grown under less favourable conditions.

The streptobacterium 13.1 and betabacterium 13.2 can hardly be termed real wine-bacteria, as they thrive best without alcohol. Yet they form an appreciable amount of acid in the presence of up to 15 % of alcohol. Growth still occurs at 21—22 %. The B- and V-bacteria, however, must be designed as actual wine-bacteria, as they thrive best in the presence of alcohol with an optimum of about 5—10 %, without appreciable differences being noted between 5 and 17—18 %, and growth still occurring at 22—23 %.

Strain V1a has grown without alcohol and the B-strains as well

made quite a good growth in its absence, although initially not the least growth had occurred, if alcohol had been lacking.

TABLE VI.

The influence of pH.

pH	Type	A1	A2	B1	B1	B2	V1
	Strain	13.1	13.2	1	2	2a	a
2.6		–0.9	–0.5	0	0.7	–0.5	–0.5
3.0		0	0	0	0	0	0
3.4		0	0	0.7	0.2	1.1	0.5
4.0		2.5	1.6	2.0	2.0	2.0	2.5
4.6		6.3	2.0	2.0	2.3	2.3	2.7
5.1		6.8	3.2	2.7	2.5	2.5	2.8
5.5		6.3	3.2	2.5	2.3	2.5	2.5
6.3		7.2	3.4	0.2	0.2	0.6	0.2

Thus the types A1 and A2 have a high pH optimum, while B and V have an optimum at about 5. Yet, a distinct growth could be noticed for all the bacteria tested at pH 2.6. Probably the decrease of the acidity at this pH is due to a decomposition of the organic acids added to the medium.

It is chiefly during recent years, in which it has been necessary partly to substitute more or less unrefined sugar syrup for refined saccharose that these bacteria have caused difficulties in the manufacturing of wine. It was therefore of interest to ascertain, whether such syrups would be growth promoting in an artificial medium when compared with refined saccharose. At the same time the addition of various substances was tested, as is shown in Table VII.

The basic medium was YYM-medium adjusted to pH 4.5 with a mixture of malic, tartaric and citric acid with the exception of one experiment the pH of which was adjusted by sulphuric acid. Sugar and syrup were inverted with HCl before being added to the medium, which further was provided with 10 % by vol. of alcohol. The media were titrated after 10 days at 30° C. The figures in Table VII are pro mille acid calculated as lactic acid.

The beet-sugar syrup in the first experiment was very impure, mostly an absolutely unrefined decoction of sugar-beet, and has generally been superior to all the added substances, as far as

TABLE VII.

Different additions to YYM-medium (cf. page 7).

Addition to the medium of	Type	A1	A2	B1	B2	V1
	Strain	13.1	13.2	1	2a	a
4% beet-sugar syrup		9.5	8.5	7.5	10.0	6.0
4% beet-sugar syrup, partly refined		7.5	6.0	7.0	7.0	4.0
2% raw sugar		8.5	7.5	6.0	8.5	5.0
2% saccharose		7.0	4.0	4.5	5.5	4.0
2% saccharose + 0.2‰ cysteine		7.0	5.0	5.5	6.5	9.0
2% saccharose + 0.2‰ cysteine germ-free filtered		4.0	4.5	4.0	5.0	3.5
2% saccharose + 0.2‰ cysteine acidified by H_2SO_4		7.5	4.0	6.0	7.5	5.5
2% saccharose + 1‰ ascorbic acid		7.5	6.0	1.5	6.0	4.5
2% saccharose + 0.2‰ cystin		6.0	6.0	2.5	5.0	4.0
2% saccharose + 0.2‰ cysteine + 15 mg $MnCl_2$/l		12.0	6.0	5.5	6.5	3.0

formation of acid is concerned. The sugar syrup in the second experiment also originated from sugar-beet, but was considerably purer, almost as clear as water. Generally it did not accelerate acid formation as vigorously as raw sugar (unrefined, but crystallized), but more so than refined saccharose. It is therefore most likely that these substitutes play a part in the greater frequency of the bacterial attacks. None of the other substances added have been able to promote decomposition of the acid as vigorously with the exception of manganous chloride, which had a most stimulating influence on strain 13.1. This, however, might be expected, as manganese especially promotes streptobacteria (28). Yet, manganese should also promote quite a lot of betabacteria, which, however, was not the case with those tested here. In most cases cysteine slightly promoted the formation of acid, whereas cystin and ascorbic acid had hardly any effect. Strangely enough filtration of the medium appeared to reduce the development greatly; most probably certain substances had been adsorbed by the Seitz filter.

Some experiments with two samples of cherry-wine, produced according to the same recipe, but at an interval of one year, proved that it must depend on a rather special factor. The oldest of these samples was filtered germfree and inoculated with B- and V-bacteria and kept at 23 and 30° C. After 18 months there was still

no trace of growth, as evidently all growth-promoting substances had been consumed. The other sample belonged to a batch a lot of bottles out of which had been spoiled by bacteria (B-bacteria).

The old sample I contained 16.87 % by vol. of alcohol and titrated 21 ml NaOH/100 ml, pH 3.83.The young sample II contained 19.66 % by vol. of alcohol and titrated 17 ml NaOH/100 ml, pH 3.91.

The two samples were treated as follows:
1) no treatment.
2) diluted with one third of tapwater: pH in I 3.85, in II 4.00.
3) 0.5 % laevulose added.
4) 0.5 % laevulose + 5 % yeast-autolysate added.
5) 5 % inverted refinery molasses from sugar-beet added.

These samples were filtered germfree and poured into sterile flasks, 100 ml in each, and inoculated profusively with precipitate from bottles of spoiled wine and then kept at 23° C. After 2.5 months a vigorous growth was noticed in sample I, where yeast-autolysate, and only a feeble growth, where inverted refinery molasses had been added; there was no growth in the other samples. As to sample II the V-bacteria had behaved similarly, whereas the B-bacteria caused vigorous growth in all the samples. The V-bacteria had formed kephir-like colonies at the bottom of the bottles — previously noticed with the B-bacteria — whereas the B-bacteria here formed a thick slimy coherent precipitate. In both cases there were long threads and chains. The wine was lactic soured and an amorphous, brownish precipitate appeared. The aroma had disappeared and an insipid odour recalling acetic acid and ethylacetate had developed.

Consequently the yeast-autolysate has induced the growth of the V-bacteria in both samples and the growth of the B-bacteria in sample I. A notable increase of acid had taken place during the growth, although much greater in sample II than in I (cf. Table VIII).

TABLE VIII.

	Wine I + yeast-autolysate		Wine II + yeast-autolysate	
	ml n/4 NaOH per 100 ml	pH	ml n/4 NaOH per 100 ml	pH
Original	21	3.83	17	3.91
+ V-bacteria	21	3.85	67	3.45
+ B-bacteria	50	3.66	100	3.28

In spite of the vigorous growth of V-bacteria in wine I, this had not resulted in an increase of acidity. On the whole, in spite of an addition of yeast-autolysate, wine I seems to be far inferior as a medium than wine II. As a matter of fact, the vigorous growth without or with only a slight formation of acid recalls the description of DOUGLAS and MCCLUNG of fortified Californian wines, attacked by „hair bacillus". There as well a slight formation of acid and sometimes gas development had occurred. Even if the latter bacterium had a lower minimum temperature than those described here, it is most probable that it also belongs to the *Bbm. longum* group.

Later experiments with wine I showed that already 1 % yeast-autolysate (with 0.5 N) promoted the growth of the B- and V-strains considerably, but 3—5 % gave maximum growth.

The influence of sulphurous acid and of pasteurisation was then ascertained for the same wine + 5 % yeast-autolysate. $K_2S_2O_5$ was added in amounts varying from 50 to 400 mg/l corresponding to about 25 to 200 mg SO_2/l. The amount of free SO_2 was not determined.

After having been kept for 2 weeks, there was growth in all samples with V-bacteria up to 300 mg $K_2S_2O_5$/l, while the B-bacteria had not grown with 200 mg $K_2S_2O_5$/l. After $2\frac{1}{2}$ months notable growth in the whole lot was observed. An amount as low as 100 mg $K_2S_2O_5$/l, however, had markedly inhibited development of the B-bacteria, whereas no less than 200 mg/l at least had this effect on V-bacteria. 300 mg still allowed some growth in both strains and they were only completely inhibited by 350—400 mg/l.

The pasteurisation tests were made in test-tubes with screw caps each containing 10 ml of medium and heated to 60° C. After inoculation the tubes were taken out at intervals of 5 minutes untill 30 minutes. It appeared that 25 minutes heating at 60° C. in the very sour wine (pH 3.83) had not the slightest influence on the rate of growth of the bacteria. After 30 minutes there was a slight delay in growth, but the bacteria were not killed off.

In practice, however, it has been proved that heating up to 80—90° C. in a plate apparatus effectively destroys these bacteria.

Accordingly the bacteria in question are extremely resistant to the methods of preservation customary in wine manufacture, a fact which marks them as dangerous infections in a wine factory.

Their optimum temperature is about 30—37° C.; the V-bacteria grew well at 10° and 45° C. weakly at 50° C., the B-bacteria weakly at 10 and 45° C.

Wine I with and without 5 % yeast-autolysate was also inoculated with strains of *Streptobacterium* A1 and *Betabacterium* A2, as well as with *Sbm. plantarum* and *Bbm. arabinosaceum* from silage. Unfortunately our stock of wine II was exhausted, so this wine could not be included in the experiment. The streptobacteria appeared to develop in wine + yeast-autolysate in the course of a fortnight, while without the addition of yeast-autolysate they developed only after 2—3 months. *Betabacterium* A2 showed no sign of growth after 3 months either with or without yeast-autolysate. *Betabacterium* from silage grew in wine + yeast-autolysate after $1\frac{1}{2}$ months, but in its absence only after 3 months. In any case the growth was only poor and the acidity had not changed. Evidently a substrate in other respects optimal, such as YYM-medium is required for a vigorous growth of these bacteria at such a high alcohol concentration and such a low pH.

As apparently the types of bacterium A1 and A2 are like ordinary strepto- and betabacteria, it seemed of interest to ascertain whether other bacteria of these genera, but of different origin, would be able to grow under the conditions offered in wine. So strains of *Sbm. plantarum* and *Bbm. arabinosaceum* freshly isolated in the laboratory in connection with ensiling experiments (Orla-Jensen *et al.* (29)) were tested in the usual YYM-medium (pH 4.34) unto which had been added 10, 15 and 20 % by vol. of alcohol. The 8 strains examined developed with 10 % of alcohol and with 15 %, only one *Sbm. plantarum* made an exception. With 20 % 3 strains grew vigorously after 7 weeks, whereas 2 grew merely slightly, and 3 not at all. Here, no difference between strepto- and betabacteria could be noted.

The optimum pH for these bacteria was about 6, but after a month at 30° C. growth although weak occurred at pH 2.4. At pH 3.1 the growth was very vigorous. Accordingly conditions prevailing in wine will not exclude these bacteria from appearing at any time as „disease-bacteria". Besides, they were all able to decompose malic acid.

It is especially in the fortified wines, which generally contain

juice from berries and the like, that the B- and V-bacteria have caused trouble. In order to study their behaviour in pure cider, investigations have been made with a type of Rhine wine, entirely produced from apples and filtered germfree before bottling. This wine contained 10.8 % by vol. of alcohol, pH 3.38. Increasing amounts of yeast-autolysate and also of $K_2S_2O_5$ and alcohol were added. Both the latter series of experiments were made with wines, supplied with 5 % of yeast-autolysate. The different types of bacteria from wine, as well as a series of strains of streptobacteria and betabacteria from silage were used as inoculum.

In the absence of yeast-autolysate neither B- nor V-bacteria had grown in the course of 4 months. The V-bacteria showed a feeble growth after supply of 1 % yeast-autolysate and an extremely vigorous one with 3 and 5 % after 1 month. The B-bacteria showed vigorous growth only with 5 % yeast-autolysate. Addition of yeast-autolysate did not act upon strepto- and betabacteria A from wine and from silage, these growing rather willingly in all media.

As to the action of $K_2S_2O_5$ all the bacteria showed feeble growth with 200 mg/l, whilst with 300 mg/l the inhibition was very marked and *Betabacterium* A2 from wine and *Sbm. plantarum* from silage did not grow at all. Another strain of *Sbm. plantarum* (No. 128) from silage still gave some feeble growth with 500 mg/l, a concentration completely inhibitory for all other bacteria. However, growth in all cases was so weak that neither increase nor decrease of acid could be ascertained and when the investigations were brought to a close after 4 months none of the cultures contained living cells.

As to the concentrations of alcohol tolerated, V-bacteria were inhibited by a percentage of alcohol exceeding 16; B-bacteria 15. So this proves cider, even when supplied with yeast autolysate, to be inferior as a medium to cherry-wine or YYM-medium, in both of which these bacteria could tolerate far higher concentrations of alcohol. The other bacteria behave similarly with the exception of *Sbm. plantarum* No. 128, showing vigorous growth with 20 % alcohol and weak growth with 22 %. This strain appears to be very resistant and would form a dangerous source of infection in a wine factory.

In order to find the reason, why the B- and V-bacteria grow so feebly in cider, a series of experiments were made, in which the vitamins from the B group (with the exception of biotin) and various

amino-acids were added to cider and juice. Also the effect of a neutralisation of the acid in these very acid media was tried (pH in the juice was 3.11, in the cider 3.38).

The result was that the common strepto- and betabacteria grew equally well with or without these substances at the different values of pH, which were tested up to pH 4.3. Yet, the growth was not so vigorous that any chemical change in the wine could be ascertained. The B- and V-bacteria did not grow with any of the substances added, whereas a slight decrease of the acidity was sufficient to produce quite a vigorous growth both in the juice and in the wine independently of vitamins and amino-acids added. An increase in pH to 3.5—3.6 was sufficient to produce a rather good growth of these bacteria, yet, a further increase did not seem to make them grow more vigorously and growth never became as abundant as in cherry-wine.

So a low pH in the juice seems to restrict the growth of the B- and V-bacteria and the other bacteria. Most probably, however, other factors will be of importance as well, as the types of fortified wines, giving good conditions for growth, generally are strongly attacked. It is to be mentioned here that by electrometric formol titration of samples I and II of cherry-wine, respectively 0.014 and 0.012 % formol nitrogen was found, while no formol nitrogen could be traced in the cider. The difference in susceptibility is, therefore, scarcely due to the content of amino nitrogen. Wine II contained considerably more alcohol but was lower in titer and had a higher pH than wine I. The better growth of the bacteria might be due to the latter fact, although it has to be kept in mind that in the YYM-medium the bacteria grew at pH 2.6.

In order to ascertain whether the yeast has any influence on the properties of the wine as far as these act upon bacteria, the following test was made.

To half of a batch of apple-juice + 0.2 ‰ $(NH_4)_2PO_4$ 15 % saccharose, to the other half 20 % raw sugar was added. After pasteurisation in portions of 250 ml all flasks were inoculated with port- and sherry-yeast respectively. From each experimental set one flask was taken out after having been kept at 20° C. for periods of 14 days, 1, 2 and 3 months, and filtered germfree through a Seitz filter. In some cases part of the wine was pasteurised at 75° C. for 15 minutes to make a comparison with the filtration. The

treated wine was kept in sterile flasks until all the samples were gathered. After transfer in portions of 10 ml into sterile tubes with screw caps, these were inoculated with the different wine bacteria. Strepto- and betabacteria from wine A grew quite vigorously in all the samples, also in the unfermented juice. The B- and V-bacteria after a month showed vigorous growth in the sample with raw sugar, having been fermented with port-yeast, kept for 3 months on the yeast and filtered germfree. In all the other experimental sets practically no growth had occurred after $2\frac{1}{2}$ months at 30° C.

Accordingly the sherry-yeast seemed to have no growth-promoting effect at all. Maybe it would have been different, if kept for more than 3 months, but in practice this is of no importance. On the other hand the existence of a difference in the ability of the various yeasts to „improve" the wine for the bacteria, might be of some practical importance.

As to the properties of the various wine samples in the course of the experiment, it can be reported that in the original juice with raw sugar pH was 3.41, the titration figure being 41.2 ml n/4 per 100 ml and formol nitrogen: 0.01 %. In the course of 3 months for both port- and sherry-yeast the pH increased gradually to 3.66. As to the former the results of titration fell to 37 ml n/4 and as to the latter to 39 ml. The formol nitrogen decreased immediately to 0.002 % and remained on that level.

The initial values for the juice with saccharose were: pH 3.24, 48.2 ml n/4 per 100 ml and formol nitrogen 0.007 %. With port-yeast pH rose to 3.53 and with sherry-yeast to 3.42, and the corresponding titers were 36.2 and 41.4 ml n/4 per 100 ml. The formol nitrogen initially fell sharply in both samples to about 0.0006 %. With port-yeast it rose after a month fermentation to 0.001 % and remained on that level, whereas with sherry-yeast it fell further in the course of 2 months to about 0.0003 %, but thereafter it rose to 0.001 %, equal to the ultimate level of the port-yeast.

The above figures prove that the raw sugar causes the pH in the cider to increase considerably compared with saccharose. This should be avoided in view of the risk of infection. So it would be more justified to acidify for instance with tartaric or citric acid before addition of the sugar. During the fermentation pH in the presence of raw sugar also rises more than in the presence of saccharose. Further the fact deserves notice that port-yeast has caused a larger increase of the pH in saccharose-wine than sherry-yeast,

probably due to a more vigorous decomposition of the organic acids. The final amount of formol nitrogen is larger in the presence of raw sugar than of saccharose, a fact worth considering too.

The above tests, however, do not furnish an explanation for the lack of growth of the B- and V-bacteria in the sample on sherry-yeast.

3) Bacteria from ropy wine.

Facts will be reported on an investigation as to the cause of a disease which now and then occurs in fruit-wine factories, the wine becoming strongly viscous, threadforming and clotted during fermentation.

The investigation bore on a wine produced from apples, rhubarb and a few raisins. The sugar was pure saccharose with 10 % of raw sugar. The disease occurred after a few days' fermentation and the fermenting juice next to a viscous consistency produced an acid smell.

Microscopical examination revealed numerous coccoid to ellipsoid cells, 2 and 2 or in short chains. Capsule staining showed them to be surrounded by a slime-cap, which was often very extended in proportion to the number of cells in the slime itself. The ,,ferments de la graisse'' described by KAYSER and MANCEAU (17) in grape-wines exactly correspond in shape to those occurring in the fruit-wine examined.

In order to isolate the bacteria, the wine was spread on C-Y-agar, provided with 0.75 % of acetic acid (pH 4.3), as they had appeared to grow very poorly without the addition of acid. The plates were incubated at 30° C. In the course of 4—5 days small (0.5—1 mm in diam.), smooth, white colonies appeared, consisting of diplococci and short strains of streptococci, which gave vigorous growth in C-Y-bouillon and YYM-medium. The media became very turbid with an abundant somewhat ropy precipitate, in which short rodforms were found together with cocci. In the YYM-medium the cells were considerably larger in size and more rodforms were noted than in the C-Y-bouillon. In stab-cultures in C-Y-agar and -gelatine with saccharose a rather strong slime formation occurred and the cocci were surrounded by a slime-cap. Gas development was noted in the ,,stab''. On the other hand it was not possible to produce slime formation in apple-juice + saccharose with or without the addition of living yeast. So it appeared that these

bacteria also grew very poorly in the pure apple-juice or cider. The addition of yeast-autolysate, however, markedly promoted their growth and it was possible to induce a be it slight slime formation, both with or without the addition of living yeast. KAYSER and MANCEAU have found that a wine rich in nitrogen is attacked much more readily than one low in nitrogen, a fact well in accordance with the low content of nitrogen in cider.

It was found that these bacteria still grow willingly at pH 3.0 in a suitable medium and like the „KAISER and MANCEAU" bacteria, are slightly inhibited by 5 % by vol. of alcohol and rather strongly by 10 %. This concentration causes the bacteria to become distinctly rod-shaped, a feature usual in strepto- and betabacteria which in higher concentrations of alcohol will tend to the formation of filaments.

The bacteria decompose malic acid vigorously and form some mannitol from laevulose. In fermentation experiments in C-Y-bouillon with 2 % of glucose, 1440 mg glucose had been fermented per ml of medium. The following products of decomposition were found:

lactic acid:	35.4 %	of	fermented	sugar
acetic acid:	4.2 %	„	„	„
ethanol	: 31.9 %	„	„	„
CO_2	: 16.8 %	„	„	„

The lactic acid formed was laevo-rotatory and apparently the organism studied is a heterofermentative lactic acid coccus, a *Betacoccus* according to ORLA-JENSEN.

In the series of sugar fermentations it was found that one out of two examined strains, next to hexoses and disaccharides, fermented xylose and arabinose vigorously, whereas the other did not ferment arabinose, although it did xylose. Both fermented melibiose, but not melizitose. According to the system of ORLA-JENSEN, the former strain is a typical *Bc. arabinosaceus*, whereas the latter should be *Bc. bovis*, which does not ferment arabinose, but sometimes xylose. As all the characters of the xylose-fermenting strains of *Bc. bovis* are identical with those indicating *Bc. arabinosaceus*, and as an equal development of both species in a same sample of wine would be hardly probable, a classification of the strains which only ferment xylose together with those fermenting both xylose and arabinose, *viz.* as *Bc. arabinosaceus*, seems warranted.

This is what HUCKER and PEDERSON (15) have done, but in view of priority they called the genus *Leuconostoc* van Tieghem and divided it in the species *L. mesenteroides*, synonym of *Bc. arabinosaceus* — but with the difference that it ferments arabinose and xylose — and *L. dextranicus*, which does not ferment either xylose or arabinose and is pro parte synonym of *Bc. bovis*, which may ferment xylose. The non-slime-forming *Bc. cremoris* (*L. citrovorus*) not fermenting either xylose, arabinose or saccharose, might appear as of no interest in this connection. Yet it may be pointed out that the existence of one or more non-slimeforming species in the genus *Betacoccus* makes the genus name *Leuconostoc* misleading, as it emphasizes the slime-formation.

Both ORLA-JENSEN (26) and HUCKER and PEDERSON state that these cocci may lengthen and HUCKER and PEDERSON point to the fact that especially in acid juices they may become pointed or even elongate into rods.

The conclusion is drawn that the slime formation in the fruit wine examined was caused by *Betacoccus arabinosaceus*. It is most probably identical with the bacterium studied by KAISER and MANCEAU.

Summary.

During recent years a typical „tourne-pousse" disease has often occurred in Danish fruit-wines of the fortified types. This disease is caused by betabacteria belonging to the group *Bbm. longum* Orla-Jensen. They are difficult to cultivate in pure culture. As initially they could not be made to grow in a medium without alcohol or in a neutral one, they are actual wine bacteria. They are extremely resistant to outward conditions, as they can stand heating up to 60° C. for 30 minutes in wine and under certain conditions grow in substrates with 23 % by volume of alcohol and at pH 2.6. SO_2 below 100—150 mg/l does not inhibit their development. Their growth is promoted by unrefined sugar syrup and raw sugar, substances which have been used quite frequently in recent years, a fact which may explain the greater frequency of this disease.

From other types of spoiled fruit-wines *Sbm. casei* and *Bbm. arabinosaceum* have been isolated. Outward conditions acted

practically in a similar way on the latter bacteria as on *Bbm. longum*. They grew best, however, in the absence of alcohol, although slight growth could be noticed at 20—22 % by vol. of alcohol. Strains of strepto- and betabacteria, isolated from silage, behaved similarly. So these bacteria are no special wine bacteria.

From a fermenting cider, which had grown slimy, *Bc. arabinosaceus* was isolated. This organism is considered as identical with „ferment de la graisse" described by KAISER and MANCEAU.

A common trait for all these bacteria is that they grew poorly or not at all in pure cider, wheras in some cases they grew well in the less acid cherry-wine richer in nitrogen and in similar wines into which berries and unrefined beet-sugar syrup had been added.

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REMARKS ON THE OCCURRENCE OF WEIL'S DISEASE IN THE NETHERLANDS

by

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In this paper the following questions are discussed:

Was the compulsary notification one of the causes of the high number of cases of Weil's disease that became known in the Netherlands during 1932?

Is there a constant difference of incidence of the disease between several parts of the country?

Is the decrease of the morbidity since 1932 the result of the measures taken in order to diminish the contact with rat-urine?

In 1923 SCHÜFFNER and KUENEN (3) communicated the result of their examination of 209 sewer-rats, trapped in Amsterdam; 27 % excreted *Leptospira icterohaemorrhagiae*, from the adults 45 %. In 1925 and 1926 the first cases of Weil's disease, in which the diagnose was based on laboratory examination, have been published in the Netherlands. A number of similar observations followed. SCHÜFFNER (4) is convinced that the occurrence of Weil's disease in our country is not new and that formerly it has not been diagnosed because the attention had not yet been called to the disease. Opposite to this, is KRAMER's opinion (1). He put forward that physicians with great clinical experience in former times never saw the syndrome.

During the period from 1925 to 4 October 1929 it is possible to estimate the number of cases (about 2 per million inhabitants yearly) from the researches of the laboratories which at that time carried out the tests for Weil's disease. After that, the disease had to be notified. Up to the end of 1947 1414 cases are registered.

The question can be posed how far the real morbidity finds expression in the registered cases. It may be presumed that the

many publications on Weil's disease have roused and maintained the physicians' interest in such a manner, that, at least typical cases, rarely will escape from their attention and will give a motive to laboratory examination. It may be, however, that not all cases are identified. In this connection I recall the form, described by MINKENHOF (2), where meningitis serosa, sometimes meningismus, is the only symptom. In the infectious ward of the Wilhelmina hospital in Amsterdam, all patients suffering from meningitis serosa are tested for Weil's disease, a positive result is not seldom. In the Netherlands febris canicola is observed very rarely. During 1946 in Amsterdam there occurred a small epidemic. These cases, registered as Weil's disease, are not considered in the following calculation.

Morbidity of Weil's disease per 1.000.000 inhabitants in the Netherlands.
1930—1947

1930	3,3	1936	7,9	1942	6,4
1931	3,7	1937	11,2	1943	6,4
1932	25,6	1938	9,8	1944	6,0
1933	18,3	1939	8,3	1945	3,6
1934	13,9	1940	7,2	1946	5,5
1935	11,4	1941	7,5	1947	6,4

After the peak in 1932, the morbidity reduced gradually, notwithstanding the fact that, in consequence of the black-out, during the winter-months of 1940—1944, particularly in the cities, the number of patients contaminated by water-accidents increased. SCHÜFFNER (5) considers the compulsary notification as one of the causes of the outstanding increase in 1932, beginning in June of that year. There is not any reason to assume that the physicians in several parts of the country, suddenly should do their duty, in contrast with the preceeding 2½ years. After all, there was always a parallelism between the number of the declarations and the positive results of the laboratory tests.

During 1932, 1933 and 1934 the provinces of South Holland, North Holland and Utrecht had an average morbidity higher than 1: 100.000 per year. Only the province of Overijssel approached this sick-rate, in the other 7 provinces it was much lower. In comparing this period with the first years after the liberation, 1945, 1946 and 1947, the morbidity in South Holland has decreased until one

seventh, in Utrecht until two seventh. In the course of years there has been little change in the province of North Holland, this may be connected with the above mentioned perfected diagnostics in cases of meningitis serosa in Amsterdam. From the other provinces, in Friesland and North Brabant the morbidity in 1945, 1946 and 1947 was somewhat higher than in the period 1932—1934, in Groningen it was unaltered and in the remaining provinces the sick-rate was lower. These calculations relate to the provinces where the patients have fallen ill. For studying the geographical spreading of the sources of Weil's disease, knowledge of the place of contamination is necessary. From a practical point of view the difference is not important; it appeared that from 100 patients with Weil's disease, only 6 had been infected outside their residence or an adjoining municipality.

In 1932 the patients to a large extent were contaminated by swimming. Measures were taken to keep the rats away from the public baths and their surroundings. Some establishments, situated in a rat-attracting quarter, have been closed or moved. The rat control also was carried out in rat-attracting industries. The number of patients who have been infected by swimming has decreased considerably since 1932. The following comparison between the years 1932 and 1947 demonstrates the reduction of the risk of contracting Weil's disease during swimming:

	1932	1947
number of days with a maximum temperature above 25° C	35	62
morbidity per million inhabitants May-October	24,2	5,9

This fall can not be explained entirely by the improvements of the public baths and their surroundings, for it appeared that the number of swimmers infected outside these establishments — that is to say on places where no measures were taken to diminish the contact with rat-urine — has dropped also, although to a less extent.

After all, it is not plausible that the fluctuation in the occurrence of Weil's disease during the last 23 years in the different parts of our country, depends from a chance of contact with rat-urine only.

Rather it seems probable that the morbidity in men runs parallel with the gravity of the epizootic among the rats. On this question, essential to our knowledge of the epidemiology of Weil's disease, certainty may be obtained by a periodical investigation into the leptospira carrier rates of rats living in several parts of the Netherlands.

Conclusions.

There is no doubt that during 1932 in the Netherlands the frequency of Weil's disease was really much higher than in the years before and thereafter. The peak can't be ascribed to the fact that Weil'disease in 1929 has been placed on the list of diseases with compulsary notification.

In comparing the years 1932, 1933 and 1934 with 1945, 1946 and 1947, it appears that the morbidity has decreased much more in the provinces of South Holland and Utrecht than in the other parts of the country.

The measures taken to diminish the contact with rat-urine only partly account for the decrease of the morbidity since 1932.

A periodical investigation into the leptospira carrier rates of rats living in several parts of the Netherlands is desirable.

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AN ENCEPHALITIS EPIDEMIC CAUSED BY THE VIRUS OF LYMPHOCYTIC CHORIOMENINGITIS

by

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The choriomeningitis virus as the cause of lymphocytic meningitis was for the first time demonstrated in Holland in 1944 by PRICK and VERLINDE (3) and later by VAN HASSELT (1). Since then we have several times detected this virus in the cerebrospinal fluid of isolated cases of lymphocytic meningitis and antibodies in the bloodserum of convalescents.

As we could also ascertain the existence of antibodies against this virus in healthy people, the serological diagnosis in a patient under examination has no value as long as the testing of the convalescent serum has not been preceded by a test of the acute phase serum.

It is known that the choriomeningitis virus can also cause meningitis in man and LÉPINE, in the chapter on choriomeningitis in: LEVADITI-LÉPINE: „Les ultravirus des maladies animales", writes accordingly: " le virus murin a été trouvé à l'origine d'épidémies d'encéphalite au Japon, de syndromes de paraplégie ayant l'allure de poliomyélite, où le virus a été retrouvé dans les sécrétions naso-pharyngées des malades. Il n'est donc plus possible de parler de la bénignité de l'infection pour l'homme".

A report of our investigation into an epidemic of encephalitis among patients and nurses of a convalescent hospital will corroborate the correctness of this view.

I. Clinical data.

The illness mostly began subacute. During a week or ten days the patients felt unwell, in the head they gradually became aware

of a strange, heavy feeling, sometimes a clear ache in the forehead and behind the eyes, sometimes in the back part of the head and the neck. In bed the patients did not make the impression of being seriously ill; also the patients themselves did not feel intensively ill. They uttered complaints, though, of being tired and lacking muscular strength, already after the slightest exertion. Also the facial expression often strikingly bespoke tiredness, was sometimes rigid; two patients showed a „mask face".

Vertigo, tendency to lipothymia and nausea, as well as a sudden decrease of sharpness of hearing occurred frequently mostly during a short period, seldom longer than one or two days till at most a week; these symptoms recurred now and then.

Complaints of paraesthesia in the extremities, neuralgic pains and pains in the joints and rhythmic muscular contractions in the legs were frequently uttered.

In nearly all cases paresis of one or more extremities, hemi- and paraplegia arose; sometimes both arms were adynamic and there was a clear paresis of one or both legs. The adynamia and the paresis were in the course of the illness mostly variable for one and the same patient; one day they were much more obvious than another. Sometimes the innervation of the tongue was disturbed. The patients showed difficulties in articulating, one of them spoke brokenly, very slowly and monotonously. In two patients disturbances of the miction could be stated, which made the impression of paresis of the urinary bladder.

The psyche was not disturbed during the illness. In many patients in the initial stage an enhanced emotionality could be observed, while in one patient a gradual change of character could be noticed.

Most patients were strikingly sleepy, in three of them one could even speak of insomnia, whereby as an inversion phenomenon agrypnia occurred during the night.

The neurological examination showed disturbances in the movements of the muscles of the eye, ptosis and nystagmus, while the reaction of the pupils on light was abnormal and convergence disturbances were demonstrable. Most patients complained of blurred eye-sight, scotomae, accomodation disturbances and were soon tired when reading. In two cases a distinct vascular injection of the bulbar conjunctiva was present.

When walking with eyes open as well as closed their walk was swaying, the Romberg test positive. Squeezing power of the hands and the power of bending and stretching the extremities were for a short or long time decreased or gone. The plantar and dorsal flexion of the feet were often insufficient. The biceps, tripecs, radiusperiost, knee and Achilles tendon reflexes were inhibited or absent, often right and left not in the same degree. Foot-sole reflexes were as a rule normal. Stiffness of the back part of the neck and the Kernig sign only occurred in a few cases.

In the cerebrospinal fluid there were few cells (12/3 at most). The reactions of NONNE and PANDY were sometimes negative, sometimes positive. The goldsol-, benzoe- and mastixsolcurves were sometimes slightly pathological.

The picture of the blood, apart from a slight to moderate lymphocytosis, showed no deviations.

The temperature curve was variable. Sometimes there was an initial top, which in only one patient reached up to 40° C., but mostly did not exceed 38.5° C. In many cases the temperature remained for weeks subfebrile, sometimes there was no rise of temperature at all. In a few patients there was during the extended subfebrile period a sudden top, during which the subjective and objective symptoms aggravated.

No acceleration or slackening of the pulse were noticed. When the temperature rose, the pulse frequency remained in proportion. Neither occurred deviations in the pressure of the blood.

Breathing remained normal, except in one patient, who one morning complained of difficult breathing and in whom also objectively a moderate dyspnea could be stated, lasting for about a week (N. phrenicus?).

Spleen and liver did not show any deviations. The urine did not contain any sugar, nor albumen.

Many patients showed bleeding in the skin, dark blue, striped bleedings, which were mostly discovered in the morning when wakening, on arms, legs, breast or back, the size of a florin to a crown. In the skin of the face and on the extremities, often near painful joints, local discolouring of the skin occurred, not sharply outlined and itching and tingling. Also changes in the colour of the skin were observed, especially for the legs, which showed an even, pale-blue tint.

One patient showed in the third week of illness a herpes-like eruption with an unusual location. On the extensor side of both upper arms on a diffuse red skin an eruption of herpes vesicles were visible. This spot was painful as in case of herpes zoster. The extension left and right was symmetrical. A month later a similar eruption arose on the palate and on the buccal mucosa.

In spite of a great uniformity the picture of the illness was pluriform in its symptoms. The course of the illness often showed acute renewed outbursts, once a recidivism or renewed outbreak was stated after six months. Apart from mild cases the convalescent period lasted for months. Up to now no symptoms of parkinsonism have been observed. Deaths did not occur.

Not a single therapeutic has had a perceptible influence (urotropine, iodine potassium, sulfa-drugs, penicillin). A paper by PLESCH (2), in which he draws the attention to the therapy of

herpes and other virus infections with the patient's own urine by way of auto-vaccination, induced us to inject a number of selected patients with their own catheter urine intramusculary 0.4 cc every other day, during 1 or 2 weeks.

Though in one patient a striking subjective amelioration could be stated the day after the first injection, afterwards no influence worth mentioning of these injections was to be observed. It was difficult to give an objective opinion at all in connection with the typical variability of the symptoms. Local or general reactions after this injection could not be stated.

II. Etiology.

The diagnosis assumed in the beginning: encephalitis (lethargica?), led us to consider the possibility of the presence of a neurotropic virus in the cerebrospinal fluid, in the blood, and in connection with certain epidemiological observations, in the nasal secretions and the throat washings.

a. Cerebrospinal fluid.

Cerebrospinal fluid was taken from five patients by cystern puncture and a number of experimental animals were injected with it (Table 1).

All guinea pigs, but none of the other animals showed a reaction on intracerebral injections. The guinea pigs died 16—21 days after the injection, after showing during 1—3 days before their death cerebral symptoms, *viz.*, spastic paralysis of the hind legs, opisthotonus and sometimes some salivation.

The brains were taken out aseptically; one half was fixed in alcohol 96 % for histological examination, the other half was kept at — 20° C. in sterile buffered glycerol 50 %, in order to make animal passages. The results of the second passage of three guinea pig brains has been taken up in Table 2.

Again the rabbits did not show a reaction, either on corneal or on interdermal injection. The guinea pigs all died after 7 days, showing the same symptoms as in the first passage. Out of the 9 inoculated mice one died after 15 days.

In the third passage only mice were injected, *i.e.* 3 with brains of guinea pig 960 and 3 with brains of the mouse that died in the first passage. Out of both series one mouse died after 13, resp. 14

Table 1.

Intracerebral injections of cerebrospinal fluid.

Patient	Day of illness	Exp. animal	Reaction	
			cerebral symptoms	died after
M.B.	1	rabbit 1743	—	19 days
		guinea pig 954	—	
		ferret	—	
		cottonrat 5	—	
		3 mice	—	
R.P.	13	rabbit 1742	—	17 days
		guinea pig 953	—	
		ferret 27	—	
		cottonrat 4	—	
		3 mice	—	
T.B.	14	rabbit 1713	—	16 days
		guinea pig 956	—	
		cottonrat 7	—	
		3 mice	—	
S.H.	25	guinea pig 955	—	19 days
		cottonrat 6	—	
		3 mice	—	
J.B.	31	guinea pig 957	—	21 days
		cottonrat 8	—	
		3 mice	—	

Table 2.

Second passage.

Material	Exp. animals	Inoculation	Reaction	
			symptoms	died after
brains guinea pig 953 (patient R.P.)	rabbit 1752	i.cerebral	—	
		corneal	—	
		i.dermal	—	
	guinea pig 960	i.cerebral	—	7 days
	3 mice	i.cerebral	—(1)	15 days
brains guinea pig 954 (patient M.B.)	rabbit 1753	i.cerebral	—	
	guinea pig 962	i.cerebral	—	7 days
	3 mice	i.cerebral	—	
brains guinea pig 955 (patient S.H.)	rabbit 1754	i.cerebral	—	
	guinea pig 964	i.cerebral	—	7 days
	3 mice	i.cerebral	—	

days. Starting from the brains of both mice regularly cerebral mice passages could be made. Provided young mice weighing about 10 grams were used, in the further passages nearly all animals died, while the incubation period could be brought down to 6—7 days, which was reached in the 6th passage. In every passage the brains of the experimental animals were bacteriologically examined; micro-organisms could not be detected. The brains of the 2nd and 3rd mouse-passage were virulent for the guinea pig (died after 7—9 days).

b. **Throat washings.**

The throat washings of 5 patients obtained by gargling in the 5th, 6th, 11th, 12th and 41st days after onset of the cerebral symptoms, were mixed and treated with 5000 units penicillin and 0.25 cc 10 % sulfadiazine per 10 cc, at room-temperature. Then a number of experimental animals were inoculated with it (Table 3).

Table 3.

Inoculation with mixed throat washings of 5 patients.

Exp.animals	Inoculation	Reaction	
		symptoms	died after
	corneal	—	
rabbit 1739	dermal	+	
	i.cerebral	+	8 days
guinea pig 950	i.cerebral	+	22 days
cottonrat 1	i.cerebral	—	
5 mice	i.cerebral	—	
2 ferrets	i.cerebral	—	

As the bacteria from the throat washings could not be removed by filtration at the spot we have applied the penicillin-sulfadiazine method.

The rabbit reacted on the corneal inoculation with a slight blurring of the cornea, visible after 48 hours, which, however, quickly disappeared. On the skin, at the spot where the material had been rubbed into scarifications, a dubious reaction arose consisting in a reddening and some swelling along the inoculation-lines, more marked than the actual traumatic reaction caused by the treatment, which, however, disappeared within a few days.

The intracerebral vaccination caused, however after 5 days a reaction, which made the existence of encephalitis probable. The animal showed atactic movements of the head and the neck and paresis of the hind legs, resulting in its death 8 days after the inoculation. Though the negative reaction of the cottonrat and the mice on the intracerebral inoculation did not make the existence of a purulent encephalitis or meningitis of bacterial origin probable, after their death a careful examination of the brains as to the presence of bacteria was made. Micro-organisms, however could not be ascertained, while a later histological examination of the brains, proved, that the animal had actually died of a non-purulent meningoencephalitis.

The reaction of the guinea pig agreed with that shown by the guinea pigs infected with cerebrospinal fluid, as far as the clinical phenomena and the period of incubation go.

The intranasal instillation of the throat washings in two ferrets was meant to detect influenza virus. This experiment, however, had a negative result.

A second passage was made with the brain of the rabbit as well as with that of the guinea pig.

Table 4.

Material	Exp.animals	Inoculation	Reaction	
			symptoms	died after
Brain rabbit 1739	rabbit 1732	i.cerebral	+	4 days
	rabbit 1741	corneal	—	
	rabbit 1733	dermal	+	
	guinea pig 964	i.cerebral	—	
Brain guinea pig 950	rabbit 1734	i.cerebral	—	
	guinea pig 965	i.cerebral	+	6 days
	guinea pig 966	i.cerebral	+	7 days
	6 mice	i.cerebral	+(1)	14 days

The animal inoculated intracerebrally with rabbit brain died after 4 days showing strong symptoms of encephalitis, the rabbit inoculated corneally, showed no visible reaction, while on the skin of the rabbit inoculated dermally a typical, herpes-like eruption arose. Guinea pig 964 did not react on the injection of the rabbit brains, guinea pigs 965 and 966, on the other hand, died after the infection with the guinea pig brain after 6—7 days under

symptoms of encephalitis; also one of the mice died after 14 days. Though already during the first passage there was a certain suspicion of the presence of herpes virus in the throat washings this suspicion became stronger during the second passage. Owing to special circumstances we were still in possession of two rabbits formerly immunized against herpes, which did not react on the intracerebral injection of the brain of rabbit 1732. The presence of the herpes virus in the throat washings of these patients can be explained, three of them having formerly had occasionally and again during their present illness eruptions of herpes simplex. They could, therefore, be considered as herpes virus carriers.

The experiments with the guinea pigs and the mice proved that in the throat washings still occurred the same virus as that which was found in the cerebrospinal fluid. Like it was the case for the cerebrospinal fluid virus, also the throat virus could be adapted on to mice, resulting in the mice dying after 8—9 days during the fifth passage. Though also the guinea pig and the mouse are sensitive to the herpes virus, it has not been possible to detect it in their brains by inoculation into rabbits.

c. **Blood.**

From two patients who had been ill for two months and showed an acute exacerbation, accompanied by a rise of temperature defibrinated blood was inoculated intracerebrally in 3 guinea pigs. Two of these guinea pigs died after 16 and 45 days respectively with cerebral symptoms. The brain of the animal that had died after 16 days, could be transmitted on to mice; adaptation of the virus such as to cause death after about 6 days, could be reached after 4 passages.

d. **Urine.**

A mixture of sterile catheterized urine of five patients who showed cerebral symptoms during 7, 12, 13, 31, and 42 days respectively, was inoculated intracerebrally into 1 rabbit, 2 guinea pigs, 2 cottonrats and 5 mice. None of the animals have reacted upon it.

e. **Identification of the virus.**

1. Control-experiments.

The animal experiments, as well as the bacteriological examination of the brains of the animals indicate that from the blood,

the throat and the cerebrospinal fluid of the patients a virus has been isolated, from the throat even two viruses, of which the herpes virus can be left out of discussion, as this had no causal connection with the epidemic and its existence may be explained by the fact that there were herpes carriers among the patients. The other virus had on first isolation only pathogenic properties for guinea pigs, but after one or two guinea pig passages it could be adapted onto mice.

Though the fact that the intracerebral inoculation of urine did not cause any reaction in guinea pigs and mice, already pleads against the animals being virus carriers, we have made control experiments along with the various passages, namely intracerebral inoculations of guinea pigs and mice with cerebrospinal fluid and blood of people from another part of the country where this illness did not occur and with sterile broth-saline solution. The fact that the animals we used for checking have remained perfectly normal, corroborated as to the absence of a latent neurotropic virus in the animals.

2. Histological examination of the brains of the animals.

The brains of all animals that died have been histologically examined after staining with cresylviolet (Nissl's method).

The rabbits which died after the infection with the virus isolated from the throat showed the picture of the herpes-encephalitis.

The guinea pigs and the mice, having been infected with the virus either from the blood, from the throat or the cerebrospinal fluid, all showed a histological picture so strikingly similar to that of the picture of choriomeningitis that already by the study of the first slides we were reminded of this disease. The meninges contained to a small extent local lymphocytic infiltrations, the chorioid plexus was, however, especially in the guinea pigs highly edematous and infiltrated with lymphocytes and large mononuclear cells. Whereas in the guinea pigs the brain tissue itself was not affected, in the mice often more or less distinct perivascular lymphocytic infiltrations were found, especially in the neighbourhood of the ventricles.

3. Neutralization experiments.

We have tested 10 sera as to the presence of neutralizing antibodies against a number of neurotropic viruses; those are the sera of:

a. 3 patients in the acute stage (1—2 days after they fell ill),
b. 3 patients, 34, 40 and 90 days resp. after the first cerebral symptoms,
c. 4 normal persons, namely 2 members of the technical staff of the home, who had never had any contact with the patients and two of us (B. and V.).

Circumstances have not allowed the examination of acute phase and convalescent sera of the same patients.

The sera have been titrated against:

a. St. Louis encephalitis virus	(LD_{50}-titer:	10^{-3})
b. equine encephalomyelitis, eastern type	(,,	10^{-6})
c. equine encephalomyelitis, western type	(,,	10^{-5})
d. choriomeningitis virus (ARMSTRONG)	(,,	10^{-4})
e. choriomeningitis virus (PRICK and VERLINDE)	(,,	10^{-4})
f. patients' virus from cerebrospinal fluid	(,,	10^{-3})
g. patients' virus from blood	(,,	10^{-3})
h. patients' virus from throat	(,,	10^{-4})

As a quantitative antibody titration was not possible at that moment, we were obliged to restrict ourselves to a qualitative examination. Of each virus strain, consisting of virulent mouse brain a suspension in saline solution was made, which was mixed with an equal part of each serum to be examined, in such a way that the final dilution of the virus amounted to 1 : 200. Further for each virus a control examination was started without serum, in which the virus dilution also amounted to 1 : 200.

After having been at room-temperature for 1—2 hours, 4 mice were inoculated intracerebrally with each mixture. The results are offered in Table 5.

The convalescent sera neutralized, therefore, both strains of the choriomeningitis virus completely, as well as the strains isolated from the patients in question. One of the acute phase sera also contained antibodies against the same virus strains, though they were not completely neutralized. It is remarkable that two convalescent sera also neutralized the St. Louis virus in some degree.

We are well aware that this experiment does not allow of any far reaching conclusions, because we were not in a position to determine the antibodies quantitatively. We think, however, that the complete neutralization of the strains of the choriomeningitis virus and of the three strains isolated from the patient by the

Table 5.

Neutralization tests.

Virus	sera										
	acute phase			convalescent			normal				virus control
	I	II	III	IV	V	VI	VII	VIII	IX	X	
St. Louis encephalitis	—	—	—	+	+	—	—	—	—	—	—
equine encephalomyelitis western type	—	—	—	—	—	—	—	—	—	—	—
equine encephalomyelitis eastern type	—	—	—	—	—	—	—	—	—	—	—
choriomeningitis (A)	—	—	+	++	++	++	—	—	—	—	—
choriomeningitis (P and V)	—	—	+	++	++	++	—	—	—	—	—
virus from cerebrospinal fluid	—	—	+	++	++	++	—	—	—	—	—
virus from throat washings	—	—	+	++	++	++	—	—	—	—	—
virus from blood	—	—	+	++	++	++	—	—	—	—	—

++ all mice remained alive; + 50 % of the mice remained alive; — none of the mice remained alive.

convalescent sera, contrary to the acute phase sera, form a rather convincing proof of the etiological significance of the choriomeningitis virus in this epidemic.

4. Cross immunity.

Two groups of 5 mice were immunized against the choriomeningitis virus ARMSTRONG strain and against the virus isolated from the cerebrospinal fluid of our patients, respectively by 3 intraperitoneal injections of formalized virus. Six weeks after the first injection the two groups were inoculated intracerebrally with the heterologous strain. All of the mice remained alive, while in two groups of 5 non-immunized mice all animals died within 9 days. Also this experiment indicates a close immunological relationship of the virus isolated from the cerebrospinal fluid with ARMSTRONG's choriomeningitis virus.

III. Epidemiology.

The convalescent home consisted of two floors. On the ground floor were i.a. rooms for the resident staff, office rooms, recreation-, conversation- and dining-rooms, library, kitchen and storehouse.

The nurses' room and those of the patients were on the first floor. The rooms were not of equal size but on an average 5 × 4 × 3.5 M. Each room contained 2—4 beds.

The population of the home varied; in Table 6 the distribution of its occupants over the various groups a fortnight after the first case of illness is taken up along with the number of cases of encephalitis in each group.

Table 6.

Group	number	cases of illness	percentage of illness
patients women	46	13	28 %
patients men	29	1 (?)	3.5%(?)
patients children (under 10 years)	7	0	0 %
nurses	14	8	55 %
maids	12	3	25 %
technical and administrative staff	18	0	0 %

It is a remarkable thing that apart from one exception in which the diagnosis was moreover very doubtful, the illness, at least in a clinically recognizable form, only occurred in women.

The age of the patients varied from 19 to 55 years, most patients however, being between 20 and 40 years. Among the children, the very young included, one of whom was even in very close contact with his sick mother for three weeks, no case of illness occurred.

The first case began in December 1946, in January 1947 10 and in Februari 14 new cases were registered.

Lodging, meals and nursing were excellent in every respect. Before the outbreak of the epidemic none of the patients were laid up. They could freely move through the building and go into the adjacent garden.

From the beginning of her illness (12-19-46) till 1-21-47, the day on which she was taken into a hospital, the first patient lay in four different rooms. During that time she was in contact with 4 ladies with whom she shared her rooms, with several other female patients who regularly visited her in her room and with a number of nurses. Two of these nurses fell ill, 13 and 19 days respectively after the first patient began to show symptoms of illness. Also other nurses and patients who had been in contact with her, fell ill, but it is equally possible that these have been infected by

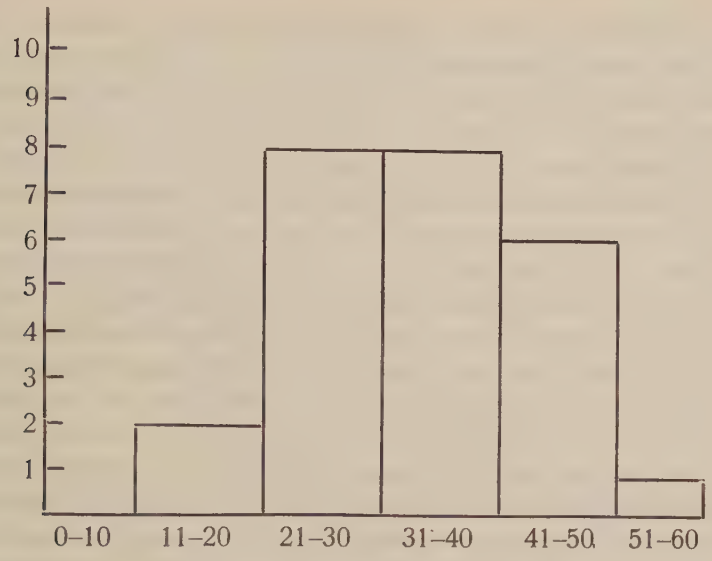


Fig. 1. Age groups.

others, *i.e.* by the second and third patient. The following three cases occurred during contacts of the second patient, *i.e.* after 11, 16 and 17 days respectively. On 18 January there were, therefore, 6 patients, who were regularly visited by the female nurses and patients who were still in good health. After that reports of new illnesses succeeded each other with intervals of 1—5 days. By frequent mutual contact in the sick-rooms infection was, therefore, possible in every direction and each new case could have more than one source of infection. An average incubation period might be concluded on 11—19 days, however, from the first six cases. Though, meanwhile, stringent isolation measures were taken by sending patients to a hospital and when due to lack of space this was no longer possible, by creating locally an isolation-room, the occurrence of a few more infections could not be prevented. Perhaps this must be attributed to the stealthy beginning of the illness, as a result of which the patients only complained when in fact the illness had been developing for some days. Also the prospect of being isolated for a considerable time induced the patients to dissimulate as long as possible.

In two cases contact either with patients or any persons still being in the incubation period, could not be traced. There had been contact, though, with nurses who also had to nurse ence-

phalitis patients, which was unavoidable in view of the many cases of illness among the nurses. It may be conceived that among these nurses virus carriers occurred without the nurses having fallen ill themselves.

Is it possible to find an explanation of the fact that the illness only occurred in women apart from one case, which was moreover doubtful? The mutual contact between the female nurses, also in the rooms of the patients, had been very frequent and covering a long period before the isolation. The maids and naturally also the nurses, were daily for some time in the sick-rooms and later in the isolation room. The virus was present in the nasopharynx of the patients, so that, according to the latest views of the droplet infection, the virus may have spread through the whole room in the form of micro-droplets and may also have settled partly on the bedding and on the floor dust. The air in the relatively small rooms, which were but insufficiently aired during the severe cold prevailing at the time must doubtlessly have contained a high virus concentration, especially during the making of the beds, the dusting and the floor cleaning. Female patients, nurses and maids have therefore in about equal measure been subject to this airborne infection. It is remarkable that the morbidity figure for the nurses is however twice as high as that for the two other groups (Table 6). Perhaps the nurses have been exposed to a mass infection several times a day, while possibly fatigue predisposed them for illness.

Naturally the male patients and the other members of the staff never entered these rooms. The only contact they may have had with healthy virus carriers, may have occurred in the passages and in the conversation hall and dining-hall, that is, in large rooms where the virus concentration, if present, must have been very low. The examination of the serum of two members of the male staff, indicates, that they have not even experienced a subclinical infection (see Table 5; sera VII and VIII). The fact proves, therefore, that only the sick-rooms have been of importance for the infection and that sickness only arose after an intensive infection by a long and (or) repeated stay in these rooms.

Though the interhuman infections indicated an aerogenic transmission of the virus, we had still to try and find the source of infection for the first patient. Though it is not certain that this patient has brought the virus into the home, it is nevertheless probable. This lady, about a fortnight before the first symptoms

of illness occurred, had paid a visit to relations for two days in a town about 30 km. from the home. She seemed not to have had any contact with sick people during her stay there, but she made the journey vice-versa in overcrowded busses. As the time between this journey and the beginning of the illness agrees with the supposed incubation period, an infection by a virus carrier, whether during the journey or when with her relations, may be acceptable, but cannot be proved.

The choriomeningitis virus is known to occur in mice. These animals had been seen but very rarely in the home. After much trouble we succeeded in catching a mouse six months after the beginning of the epidemic, but the virus could not be traced in this mouse.

Perhaps the first patient, though probably indirectly, has been the cause of the infection of a woman living in another part of the country. The two-year-old son of the former was before and during the first three weeks of her illness in the home and nearly always in her room. On 8 January 1947 he was fetched by a sister-in-law of the patient, who took charge of him for five weeks in her own house, more than 100 K.M. from the home. When the child had been with her for three weeks, she also fell ill showing symptoms rather closely agreeing with those shown by the patients in the home: curious headache, pain in the muscles, pain in the back, especially in the sacral region, fever, ptosis: she recovered after an illness of 4 or 5 weeks. This seems to be a mild form of the illness described by us. We are inclined to assume that the child has played the part of transmitter of the illness without having fallen ill itself.

IV. Discussion.

Though epidemics of choriomeningitis are known, as far as we could gather, an epidemic in a closed community with such clear interhuman infections, has never been described. No wonder that for the first patients, who indeed showed alarming cerebral symptoms, also in connection with its occurrence in winter, we thought of encephalitis lethargica. In course of time, however, the disease was more protracted than severe. The fact that with such a large number of patients not any death occurred, began to form an indication against encephalitis lethargica as well as against the various forms of epidemic encephalitis type B, which all have a

high morbidity figure (the letality varies from 30—90 %). We had, however, also to consider that a virus of relatively low virulence might be in cause. The protracted process, sometimes with acute intensifications, though none of the patients succumbed, made us fear for the worst as regards any sequelae, *i.e.* parkinsonism. When it had become certain that the choriomeningitis virus was the cause, our prognosis seemed to us much more favourable. Deaths as well as sequelae are exceptions in this disease.

This choriomeningitis epidemic is in many respects different from the classical form.

In the first place the epidemiology deviates with respect to the great number of interhuman infections. We must, however, remark, that circumstances have promoted this course of things greatly. In the course of 1947 in the Institute of Preventive Medicine the ARMSTRONG virus could be demonstrated in the cerebrospinal fluid and anti-bodies in the blood of several sufferers from typical choriomeningitis and in cases showing more the picture of encephalitis. These were isolated cases in the most divergent parts of the country, while not any connection with the epidemic described above could be ascertained. From this it appears that the virus is now quickly spreading in Holland and that the distribution of the illness all over the country does not deviate from that in other countries.

This epidemic in a closed community shows that it is not only to mice we must ascribe a predominant significance as transmitters of the virus, though as natural virus reservoirs they should not be neglected.

In the second place the course of the illness is such as to deviate from the classical picture. In general choriomeningitis is described as a benign illness of short duration. Here we had to deal with a cerebral form lasting for months, not only for this local epidemic, but also in many isolated cases. For Holland, however, it is still a new disease. Before 1944 its occurrence had never been stated, and probably it did not occur. One of us (V.) has examined a number of sera of people who had formerly suffered from a lymphocytic meningitis of unknown origin, but only in one case neutralizing antibodies against choriomeningitis could be stated and that in a patient who had been ill after 1944, the year in which choriomeningitis was stated for the first time in Holland. Antibodies remain demonstrable in the blood for years; therefore, if the virus should

have circulated among the Dutch population before 1944, antibodies would have been found to a higher percentage. In America in 12 % of the population these antibodies occur in the blood!

Probably we must assume that the virus was not imported into Holland until the end of the second world war. There is not yet a population immunity and therefore there is a possibility that the comparatively serious cerebral form still predominates. As the virus is spreading and the population will attain a certain degree of immunity the picture of the illness may develop favourably.

As regards the stating of the virus we will emphatically point to the desirability to transmit the material of the patient preferably at once on to animals, or, if this is not possible, to freeze the material and have it sent to the laboratory where the virus examination is to take place ice-packed. It repeatedly appeared that if the material had been forwarded without such precautions, the virus could not be traced it being weakly resistent, at normal temperatures.

When later the bloodserum tested for the presence of neutralizing antibodies, in many of these cases it could be proved that an infection with the ARMSTRONG virus had actually been experienced.

A clear example of the importance of treating the material quickly is the following. In the cerebrospinal fluid of a girl suffering from lymphocytic meningitis, by inoculation of mice and guinea pigs more than 24 hours after the lumbar puncture, no virus could be demonstrated. When, however, some time later, a fresh sample of cerebrospinal fluid could be inoculated within half an hour after the puncture, it was possible to demonstrate the choriomeningitis virus. Further our examinations have shown the necessity to make inoculation experiments not only with mice, but also with guinea pigs. It seems also to have been the experience of others that some strains of the choriomeningitis virus, directly from man infect guinea pigs more readily than mice. With the first strains isolated in Holland this was not the case; they could be readily transmitted on to mice. Whether at present our mice are less sensitive to the virus than formerly, or whether these virus strains possess other properties, is a question we are unable to answer. The animals do not posses an immunity, for the virus can be easily adapted on to them.

Summary.

In the winter of 1946/1947 there prevailed an epidemic of a subacute benign encephalitis showing no sequelae in a convalescent home. After a promodal stage of 7—12 days paresis of the extremities and the eye muscles arose, ptosis, accomodation and convergence disturbances, paraesthesia, which symptoms were mostly accompanied by striking sleepiness and abnormal reflexes. Meningeal symptoms occurred but rarely. Many patients showed bleedings in the skin. The original diagnosis: encephalitis lethargica had to be abandoned when it appeared possible to isolate from the cerebrospinal fluid, the blood and the throat washings a virus which at first only could be transmitted on to guinea pigs, but which after two guinea pig passages also affected mice and which was identified as choriomeningitis virus. The virus isolated from the patients as well as two strains of the choriomeningitis virus were completely neutralized by the convalescent sera, not or incompletely by the acute phase sera. Also from cross-immunity tests it proved to be closely related to the choriomeningitis virus.

The disease only occurred in the female patients (28 %), the nurses (55 %) and the maids (25 %), not in the male patients and the technical and administrative staff. There was not any indication that mice have been the source of the infection, but all cases could be explained by contact (droplet) infection, first because the virus could be demonstrated in the throat, second in view of the initially close mutual contact among the female population of the home. The way in which the infection has been introduced into the home, could not be shown.

The character of the virus deviated in so far from the classical choriomeningitis virus that it was not possible to infect mice with material from man; it was possible, however, after guinea pig passage. Of late we have isolated a few strains of the choriomeningitis virus, which affected guinea pigs more easily than mice. Also the epidemiology deviated from that of choriomeningitis, but we may assume that the special circumstances in the home contributed here unto.

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(From the Farm Laboratories of the National Institute for Medical Research, Mill Hill, England).

THE QUESTION OF THE PRESENCE OF INFLUENZA VIRUS IN THE FAECES OF MICE AFTER INFECTION AND FEEDING WITH THE VIRUS

by

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Since the discovery in this laboratory of human influenza virus some 14 years ago only one account of the recovery of influenza virus from the faeces of infected animals has been reported in the literature. This report was published in the U.S.A. in 1941 by SARRACINO and SOULE (4), who stated that they had succeeded in identifying influenza virus (PR8 strain) in the faeces of infected mice three days after these mice had been inoculated intranasally with a mouse-adapted PR8 strain. The presence of influenza virus in the faeces of infected animals may be of great importance for the epidemiology of influenza and a further investigation into this subject seemed necessary.

Recently Dr. C. H. ANDREWES, in a single experiment, was unable to demonstrate any surviving virus in the faeces of mice which had been inoculated intranasally with virulent mouse-lung filtrate (PR8) three days previously. Taking this into consideration, our first attempt, therefore, was to repeat in exactly the same way the experiments of SARRACINO and SOULE who, as mentioned above, had been successful.

In all our experiments we used a mouse-adapted PR8 strain, which had been studied very thoroughly in this laboratory and which was able to kill mice within 3—4 days after intranasal

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inoculation with a 1/1000 dilution. A group of six mice was inoculated intranasally with 0.05 cc of a 5 % PR8 virus filtrate. Three days after the inoculation, the animals were placed in a clean, sterile cage. Faeces, collected immediately upon defaecation, were placed in a physiological salt solution and thoroughly emulsified. Enough ether was added to make a 10 % mixture which was kept in the cold room for 36 hours. The ether was then removed by evaporation under reduced pressure and the residue inoculated in the usual way into two groups of six mice. The first group was killed after three days and to our satisfaction their lungs showed lesions which resembled the usual influenza lesions. The average consolidation shown in these lungs was estimated at 4/8. The numerator being the amount of lobes showing consolidation and the denominator being the total amount of lobes in the lungs of one normal mouse. Ordinary bacterial contamination was excluded by control agars. Atypical mouse-pneumonia virus, which sometimes crops up in our laboratory mice, was also excluded by staining smears of the lung material repeatedly for elementary bodies. The lung suspension so tested was then used for a neutralization test with the specific antiserum, in order to prove that the lesions were caused by PR8 strain. Again two groups of six mice were inoculated intranasally with the last suspension. The first group received mixtures of equal parts of the lung suspension and IH5 serum, which is the specific horse antiserum against PR8 used in this laboratory. This mixture was allowed to stand at room temperature for one hour and then inoculated. The second group of mice acted as a control group and was inoculated with a mixture of equal parts of lung suspension and serum broth left standing for one hour at room temperature. The dilution of the lung suspensions was 1/100. After 10 days both groups of mice were killed, but to our surprise neither group showed any significant lung lesions.

In the meantime the second group of mice, which had received the original faeces-suspension, had been killed ten days after the inoculation and this group also did not show any significant lung lesions. So the agent present in the faeces, caused an average lesion of 4/8 three days after inoculation, but remarkably enough faded away ten days after. This agent was unlikely to be our original PR8 virus, which shows 4/8 lesions after three days and kills the mice within ten days with almost complete consolidation of the lungs. Moreover, the negative result of the neutralization test was

a definite proof that the agent present in the faeces was not the original PR8 virus. An attempt to reproduce our original lung lesions of 4/8 by repeated passages through other groups of mice remained without any result. We repeated the above-described experiments three times but were never able to establish the original PR8 virus from which we started.

Our failure to repeat the experiments of SARRACINO and SOULE, who only used the ether treatment to identify their virus, made us decide to try other methods for the selective extraction of influenza virus from infected material. In a large-scale attempt to identify influenza virus in the faeces of infected mice we thought it also worthwhile to investigate faeces from mice, which had received the virus by mouth instead of by the intranasal route. This was at the same time an attempt to find out whether the virus could survive passage through the alimentary canal. To have more chance of detecting the virus, we used in all our methods faeces collected 20 hours, 2 days, 3 days and 4 days after the original administration of the infective agent.

Methods.

Before using the methods described below, we first tested them using virulent PR8 virus suspensions, in order to make sure that we were using really effective methods for the extraction of influenza virus.

1. The first method tried by us was a method described by HERRARTE and FRANCIS (2), which was used by them as a successful method for the extraction of a poliomyelitis virus from contaminated material. In this method the virus is, after centrifuging, mainly precipitated in a solid middle layer, formed by the protein-precipitent acetone and has to be dissolved in an alkaline buffer of pH 7.9 afterwards. To a faeces suspension equal parts of chilled acetone and ether are added, the mixture shaken and then centrifuged in the cold; this was essential, as at higher temperature acetone may destroy the virus. The ether layer separated on top and a small layer of solid material in the middle. After evaporation, the solid middle layer was resuspended in an alkaline buffer of pH 7.9 and inoculated in the usual way.

2. Selective Ultrafiltration. Gradacol membranes (Elford) of A.P.D. 0.7 μ were used to select the virus from the

faeces suspension. A faeces suspension was centrifuged and the supernatant fluid carefully filtered through membrane and then inoculated.

3. Virus-concentration method by adsorbing on chicken erythrocytes.

Hirst (3) first described this method of absorbing influenza virus on erythrocytes for concentrating influenza virus. After a faeces suspension had been filtered through membrane, the ultra-filtrate was mixed with a 2 % suspension of fresh chicken erythrocytes and allowed to stand at room temperature for two hours. The volume of ultrafiltrate was 4 times the volume of red cell suspension.

Hereafter the mixture was centrifuged and the red cells, which should have absorbed the virus by now, suspended in half their volume of saline. This suspension was left for three to four hours in an incubator at 37° C. and then centrifuged. Now, however, the red cells were discarded as the virus should be in the supernatant fluid. In the supernatant fluid the virus should have been concentrated ten times, and this was used for inoculation.

Experimental.

All mice used were Swiss mice, bred at the Medical Research Council from laboratories at Mill Hill. We collected faeces in sterile cages from mice which had either been inoculated intranasally, or had been fed with PR8 virus previously. The food used was bread, impregnated with a 1/100 PR8 virus suspension. PR8 virus suspended in serum broth was refused by the mice, but suspended in sterile water was taken without difficulty.

All mice which had been inoculated intranasally with the 1/100 PR8 virus suspension showed typical lung lesions and died three to five days afterwards. No mice, however, fed with the 1/100 PR8 virus suspension became ill nor showed any lung lesions when killed three to five days afterwards.

All faeces used were made up in a dilution of 100 mg per cc which was the strongest concentration suitable for use. For convenience we distinguished two types of faeces as type A and type B. Type A were collected from mice which had been inoculated intranasally 20 hours, 2 days, 3 days or 4 days previously with a 1/1000 PR8 virus suspension. Type B were collected from mice which had been fed 20 hours, 2 days, 3 days or 4 days previously with bread,

impregnated with a 1/100 PR8 virus suspension. Table 1 gives a survey of some of the experiments performed.

Table 1

Faeces collected from infected mice	Inoculated with faeces A		Inoculated with faeces B	
	6 mice average lung lesion after 3 days	6 mice average lung lesion after 3 days	6 mice average lung lesion after 3 days	6 mice average lung lesion after 3 days
1. Faeces collected 20 hours after infection, treated with method 1.	3/8	1/8	3/8	1/8
2. Faeces collected 20 hours after infection, treated with method 2.	4/8	2/8	3/8	1/8
3. Faeces collected 2 days after infection, treated with method 1.	3/8	1/8	4/8	0/8
4. Faeces collected 2 days after infection, treated with method 2.	4/8	1/8	3/8	1/8
5. Faeces collected 3 days after infection, treated with method 1.	2/8	0/8	2/8	0/8
6. Faeces collected 3 days after infection and treated with method 2.	4/8	0/8	4/8	1/8
7. Faeces treated with method 1 4 days after infection.	3/8	0/8		
8. Faeces treated with method 2 4 days after infection.	3/8	0/8		

In every group of mice ordinary bacterial contamination and infection with atypical pneumonia virus were excluded by control agars and staining for elementary bodies, as described above. Moreover, of every group of mice which was killed after 3 days, lung suspensions were inoculated into new groups of mice in an attempt to increase the lung lesions. However, here also all lesions faded away, and in no case could we transmit the original lung lesions.

At the same time the same lung suspensions were used to put up neutralization tests with the PR8 antiserum, which, in all instances, were completely negative.

Having found it impossible to extract the original virus with these last two methods, we had as a last resort our third method, the virus concentration on red blood-cells, which was by far the most sensitive one. We performed exactly the same experiments with this method as we did with the others, but now we were unable to find any lung lesions at all, even three days after infection, the period when we were sure to find lesions with our previous methods. The failure of this last method, which was exclusively a method for concentration of influenza virus, was for us the strongest evidence that presence of this virus in the faeces, we investigated, did not account for the lesions we found.

Finally it has to be said that in the course of our experiments we were unable to infect mice with the influenza virus by intestinal inoculation, as was mentioned above. This showed again that, as far as we know at the present time, the only tissues in which influenza virus can survive are the respiratory organs and, under exceptional conditions, the brain (STUART-HARRIS (5)).

Discussion.

The failure to isolate any PR8 virus from the faeces of infected mice, in spite of the more sensitive methods we used in comparison with SARRACINO and SOULE who only used the simple ether treatment in preparing the faeces, may have various explanations. One of them may be that SARRACINO and SOULE, before identifying their virus with neutralization tests, had to accomplish five serial passages of their recovered agent, in order to establish it in their mice. During these repeated passages a laboratory contamination may easily have occurred. This danger of laboratory contamination

in influenza virus research is always present, especially when minute amounts of virus have to be identified by the technique of repeated passage. This danger for influenza workers has been investigated extensively by ANDREWES, GLOVER, HIMMELWEIT and WILSON SMITH (1) in a report in which they describe fourteen incidents occurring in ten different laboratories, where there was strong suspicion that influenza virus had acted as a contaminant.

Among other explanations for our failure may be the fact that, though we duplicated SARRACINO and SOULE's experiments and used exactly the same method of treating the faeces, it is not possible to use exactly the same laboratory animals under the same conditions; as, for example, variation in feeding can never be avoided. This as yet unknown condition, may well be able to throw more light on the difference in behaviour of some influenza virus strains as shown in different laboratories.

Summary.

Repeated attempts to duplicate SARRACINO and SOULE's experiments to identify influenza virus in the faeces of infected mice proved to be a failure. Other, more sensitive methods for extracting minute amounts of influenza virus from contaminated material are described, but they also proved incapable of extracting influenza virus from the faeces of infected mice. Attempts to infect animals by the intestinal route produced no result and at the same time we failed to prove that the influenza virus can pass the alimentary canal without being destroyed.

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(From the Laboratory of the Waterworks, Rotterdam).

IS IT JUSTIFIED TO USE LACTOSE BROTH FOR THE DETECTION OF *B. COLI* IN THE PRESUMPTIVE TEST OF ROUTINE WATER ANALYSIS?

by

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For the detection of *B. coli* by the ,,presumptive test" in the Anglo-Saxon countries and in U.S.A. it is customary to use lactose broth or lactose-peptone solution, whilst in England a further addition of bile is usual. Although I can merely offer sparse data, I hope, however, to prove in the following, that this method may not be entirely satisfactory. In 1935 (3) I compared the results of coli tests in raw and purified river water by using either the Eijkman test or a completely synthetic medium, made up as follows: 1 % d glutamic acid, 1 % ammonium lactate, 0.3 % K_2HPO_4, $^1/_2$ % NaCl and 1 % glucose, the glutamic acid having been neutralized with NaOH. The most favorable conditions are realized by culturing in completely filled stoppered bottles at 42—43° C. Growth and gas formation after 24 or 48 hours point to *B. coli*. The medium is very selective, as it does not allow the development of Streptococci, *Proteus* species, or *Bac. aerosporus*. Butyric acid bacteria usually do no occur earlier than after 48 hours and their development may be largely prevented by a low initial pH. By streaking on eosine methylene-blue plates the fermenting organisms can be identified. As a rule *B. coli* will develop with its typical dark colonies with metallic lustre; now and then however, the larger, more mucoid colonies with a light margin and dark centre of *B. aerogenes* will occur. Very frequently, however, in surface water carrying a fecal pollution fairly flat, translucent, white colonies will occur, formed by bacteria with the following properties: Gram —, short motile rods, fermenting glucose but not

lactose, methylred +, Voges Proskauer —, indole +, citrate —, whilst gelatin is not liquefied and no spores are formed.

KRUGERS DAGNEAUX (6) and also SCHAEFFER (9) isolated this bacterium from water and probably EYKEN (2) as well has encountered them.

The same bacteria, however, occur also regularly in water which has passed slow sand filters, provided with fresh sand. The bacterial control of such water preceding the connection of the filters to the pure water canals, learns that first of all streptococci disappear, then the true *B. coli*, whilst the above bacteria disappear merely slowly. Also in the control of the chlorinated water from newly laid water pipes a similar phenomenon occurs. So the organisms are also more tolerant to chlorination. When in such cases lactose broth would be used as an enrichment medium for *B. coli*, the presence of these bacteria would be overlooked and the freshly sanded filters or the freshly laid pipes would be put to use too early. In fact it will appear from the following that the latter bacteria are true *B. coli* which have lost the property of fermenting lactose and that this property may be restored by altering the growing conditions.

In order to restore the formation of gas (from glucose) by non-fermenting paratyphoid strains POT (8) carried out a series in Ca-formate-peptone water. Vitamins and other growth promoting factors may however be of significance for lactase formation.

In our investigations we have made use of 5 one-cell cultures, originating from lactose non-fermenting strains isolated in the routine water control. Dr. S. L. SCHOUTEN at Utrecht had the kindness to make these one-cell cultures for us. Culturing took place in completely filled stoppered bottles at 37° C., whilst always after 24, resp. 48 hours growth and gas formation were read. Moreover eosine methylene-blue agar was streaked in order to examine growth on this medium.

Culturing took place in the following nutrient media:

1*a*. peptone-lactose (1 % peptone-Poulenc, 0.5 % lactose, 0.5 % NaCl, 0.025 % K_2HPO_4).
1*b*. peptone-lactose as 1*a* + 0.02 % vitamin C.
2*a*. bacto-tryptone-lactose (3 % bacto-tryptone, 0.4 % K_2HPO_4, 0.15 % NaCl, 0.5 % lactose).
2*b*. Casein-tryptophane-lactose ½ %.
2*c*. casein-tryptophane-lactose 2*b* + an equal volume of sterile milk.

The sterile solutions were always shortly boiled before use and then carefully cooled down in order to reduce the amount of oxygen solved at the start as much as possible. The results are taken up in the Table 1.

Table 1.

	Nutrient medium	strain no. 1		strain no. 2		strain no. 3		strain no. 4		strain no. 5		Total number of transfers
		G	L	G	L	G	L	G	L	G	L	
1*a*	peptone-lactose	—	—	16	16	—	—	21	22	—	—	25
1*b*	peptone-lactose + 0.02 % vitamin C	—	—	5	5	—	—	21	21	18	18	25
2*a*	bacto-tryptone lactose	—	—	—	—	—	—	—	—	6	—	6
2*a*	casein-tryptophane lactose	2	—	1	—	6	—	2	—	3	6	9
2*c*	casein-tryptophane lactose+ milk	8	8	3	3	—	—	5	8	3	4	8

The columns G mark the number of transfers after which gas formation could be observed, the columns L the number of transfers needed for typical growth on eosine methylene-blue agar.

The series 1*a* shows that a daily transplant in lactose-peptone induces gas formation by strain 2 after 16 transfers and by strain 4 after 21 and that after as many as 25 transfers the remaining strains do not show any such tendency.

The experimental series 1*b* shows the favorable effect of the supply of 0.02 % vitamin C on strains 2 and 5, hardly any action on strain 4.

In the experimental series 2*a* and 2*b* bacto-tryptone has been compared with a solution of casein-tryptophane, prepared as follows:

In 500 ml tapwater 40 g finely powdered casein and 4 g Na_2CO_3 (water free) are digested till the complete solution of all casein as sodium caseinate. After cooling down to 37° C. 1 g trypsine (powdered) is added and shaken

till the complete solution of all trypsine. 3 ml chloroform is added and the solution is incubated during at least 48 hours at 37° C. After evaporation of the chloroform on a sandbath the solution is cooled down and filtered; the filtrate is made up to 2 l after a sterile solution of 4 g K_2HPO_4 has been supplied and the pH is brought down with n HCl to 7.0.

This casein-tryptophane solution was chosen, because the indolylacetic acid active as a growth promoting substance (and intermediate product in the breaking down of tryptophane) might be significant for the formation of lactase.

Bacto-tryptone was merely active on strain 5. The formation of gas did not always coincide with a typical growth, a fact hard to explain. When, however, some sterile milk had been supplied, such as in the experimental set 2*c*, typical growth did occur on the eosine methylene-blue plates. It is concluded that the vitamin B complex as well appears to act favorably on the formation of lactase. When the growing conditions of the lactose non-fermenting strains of *B. coli* are suitably altered, the lost property of lactase formation may be restored to all of them.

Not only from fecally polluted river water and from sewage lactose non-fermenting *B. coli* may be isolated, but also from fresh feces. When a small quantity of feces is streaked on eosine methylene-blue agar repeatedly during a long period, it appears that in feces from persons in normal health lactose non-fermenting *B. coli* periodically predominate especially in summer and autumn. We mostly encountered them in specimens from vegetarians and it may occur that patients, who have to keep to a strictly vegetarian diet excrete none but lactose non-fermenting *B. coli*.

STUART and co-workers say in their paper on: The biochemical and antigen relationships of the paracolonbacteria (10) ,,coliform cultures fermenting lactose slowly or not at all but normal in other respects, were frequently encountered", and ,,aberrant coliforms are frequently isolated from such sources as water with an excellent sanitary survey. On the other hand aberrant coliforms are often isolated from fecal specimens especially during gastro-enteritis epidemics".

Taking the above facts into account it seems to me that lactose is not a suitable source of carbon for the detection of *B. coli*, no more so than it is in the presumptive test. It has to be substituted by glucose. The use of glucose implies, however, that in a general sense all highly splitted proteins, termed as peptones, have to be

deemed unsuitable sources of nitrogen in enrichment tests for *B. coli* and this for the following reasons.

By using peptones along with glucose the conditions of growth for other organisms competing with *B. coli*, such as butyric acid bacteria, streptococci and others are rendered so favorable that *B. coli* will often be overgrown or killed off by the metabolic products of the former (lactic or acetic acid and especially H_2O_2 by streptococci, butyric acid bacteria etc.). A synergetic fermentation brought about by bacteria other than *B. coli* is also possible. It is common practice to supply various dyes or other toxic substances (in France phenole, test of VINCENT) which are deemed to inhibit the competing organisms and not *B. coli*. It may easily be conceived, however, that weakened *B. coli* strains (f.i. by chlorination) will be inhibited as well and thus will escape detection. In order to prevent a quick increase of pH, buffering substances are sometimes added, such as phosphates (improved Eijkman test), but evidently these are none but palliatives. A better method consists in the reducing of the source of nitrogen to a level which will suffice to *B. coli* and not so to the competing organisms. The first to realize this idea were KAUFFMANN and SMIT (5), who made use of d glutamic acid, using lactose as a source of carbon. Experiments carried out nearly simultaneously by me learned that also ammonium lactate might serve excellently, using glucose as a source of carbon. The aim is reached better still by using mixtures of Na-glutaminate and ammonium lactate and in 1934—35 I have run many experiments for the determination of the proper ratio under various conditions. In different seasons ammonium glutaminate and ammonium lactate proved to be most satisfactory in the ratio 2 : 1 or 1 : 1 or 1 : 2, but generally speaking the ratio 1 : 1 was the most suitable.

It appears from an investigation of BEEUWKES and BANNINK (1), that also paratyphoid strains A, B, C, and Suipestifer thrive well in this medium and form gas and acid. These pathogenic intestinal bacteria, however, as well as dysentery bacteria may be easily overgrown by *B. coli*, present always in greater numbers. In 1939—40 I mentioned (4) as further advantages of this nutrient medium: „Ammonium lactate is very cheap and is obtainable as an 80 % solution. It is fairly readily utilised as a source of nitrogen by *B. coli*, although a better result is obtained by the addition of d glutamic acid. The mixture of these two nitrogenous compounds

has a distinct advantage over peptone, as they are both chemically pure substances of known composition, and difficulties arising from the use of different brands of commercial peptone, of unknown composition, may thus be avoided. During four (nowadays twelve) years employment of this medium I never encountered organisms causing a false presumptive test: Streptococci, *B. proteus* and *Bac. aerosporus* alike failing to grow."

In enrichment tests for *B. coli* the oxygen content of the water sample is very important. If it is high, f.i. 10 mg/l or more, the fermentation will generally be impeded, as it only begins, when all oxygen has disappeared from the medium. A favorable circumstance is, however, that *B. coli* is facultatively anaerobic, so that it will thrive in a medium with oxygen, whilst butyric acid bacteria will be inhibited by the least trace of oxygen. When the removal of oxygen is furthered by chemical means, such as the introduction of iron nails in the fermentation bottles, the fermentation rate of *B. coli* will be increased, risking, however, that by a too rapid disappearance of oxygen the butyric acid fermentation will predominate. A suitable fermentation medium for *B. coli*, however, must meet the demand that it may be brought quickly enough in a reduced condition such that the fermentation may occur. It is desirable to bring surface water quickly up to 42—43° C., as aberrant groups of the coli group, such a *B. aerogenes* and strains of *B. coli* adapted to lower temperatures will then cause less trouble.

The preparation of the nutrient medium is as follows:

1 % glucose, 0.5 % d glutamic acid, 0.5 % ammonium lactate, 0.5 % NaCl and 0.3 % K_2HPO_4.

Solution 1.	Solve in 350 ml hot tapwater:	
	d Glutamic acid	25 g
	Ammonium lactate 80 %	31 g
	K_2HPO_4	15 g
	to reach pH 7.0 add 31 ml 5 n NaOH	
	to reach pH 6.0 add 23 ml 5 n NaOH.	
Solution 2.	Solve in 150 ml tapwater:	
	Glucose	50 g
	NaCl	25 g

Sterilise solutions 1 and 2 separately in the steamer, mix before use and dilute 1 : 10 with the water sample. Cultivate in completely filled stoppered bottles at 42—43° C.

Postscript.

The usual practice in the coli test at Rotterdam is to streak all fermentations obtained after 24 and 48 hours on eosine methylene-blue agar, for the mere ascertainment whether the fermentation is due to a coliform strain or to butyric acid bacteria. In the latter case nothing will develop on the plate. Any further differentiation of the coli strain in daily water control is deemed superfluous, as this would retard the conclusion as to the quality of the water.

Doubtlessly the composition of the above medium for *B. coli* might be altered or improved. It may easily be conceived that the addition of one or other chemical compound might render the medium still more selective for *B. coli* and I can entirely agree with the words of Winslow (11) part of which I will cite: ,,My plea is for a fundamental reëxamination of this whole problem the colon (coliform) test (in its standardized form) with an open mind, and with a clear view of the practical results to be obtained and of the margin of error associated with their interpretation. The pressing need for economy and efficiency makes such a reëxamination peculiarly imperative at the present time. If we can cut down the cost of an examination we can make more examinations and a fuller measure of control should vastly overweigh any 5 or 10 percent loss in accuracy".

A paper of Levine (7): ,,Difficulties and limitations with the bacteriological examination of water" points in the same direction.

I am greatly endebted to Dr. E. L. Molt, bacteriologist at the Municipal Waterworks, Rotterdam, who ran the experiments for the restoring of the property to hydrolise lactose.

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(Biophysical Research Group under the direction of Prof. Dr. Ir. A. J. KLUYVER, Delft, and Prof. Dr. J. M. W. MILATZ, Utrecht)

PHOTOTAXIS IN THE PURPLE BACTERIUM *RHODOSPIRILLUM RUBRUM*, AND THE RELATION BETWEEN PHOTOTAXIS AND PHOTOSYNTHESIS

by

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§ 1. INTRODUCTION.

We owe most of our knowledge of phototaxis in purple bacteria to the investigations of ENGELMANN (8, 11, 12). ENGELMANN seems to have been the first author who demonstrated purple bacteria to be capable of phototactic reactions. These organisms proved to accumulate in the light part of an only partially illuminated microscopic field. This phenomenon, already described by COHN (5, 6) in relation to other phototactic organisms (flagellates, green algae), was to a certain extent elucidated by ENGELMANN. He observed that a sudden reduction in the intensity of the light caused a so-called ,,shock reaction'', as a result of which the direction of movement was changed. On the other hand, a sudden rise of the light intensity appeared to have no phototactic effect. The organisms' property of showing a shock reaction on a sudden reduction of the light intensity prevent them from crossing the boundary between light and dark, after having entered the light spot. Conversely, the organisms are not prevented from entering the light spot from the dark. Consequently, a light zone acts like a trap.

The explanation of ENGELMANN with regard to the accumulation of phototactic organisms in an illuminated area excludes any influence exerted by the direction of the light on phototaxis. In the case of the purple bacterium investigated by ENGELMANN (*Bacterium photometricum*), an influence of light direction on phototaxis could only be established, if there was also a gradient of intensity, *e.g.* in a divergent beam of light. In parallel light no

phototactic reactions were observed. As the later investigations of MOLISCH (23) and of BUDER (2) have completely confirmed this in the case of other purple bacteria, it was not necessary in our experiments to take into consideration a possible influence of the light direction otherwise than in the sense indicated above.

ENGELMANN's brilliant investigations regarding the distribution of phototactic sensitivity of *Bact. photometricum* have also stood the test of time (10, 11, 12). In sunlight, in gas light, and in incandescent light the bacteria collected in bands in the infra-red region between 9000—8000 Å, in a somewhat less distinct band in the yellow at about 5900 Å, and in a still less distinct band in the green between 5500—5200 Å. ENGELMANN also observed that a thin layer of bacteria showed about the same absorption spectrum (maxima at 8500, 5900, 5300, and 5000 Å) as the spectral distribution of phototaxis in the micro-spectrum. In other organisms he established a similar relation between the spectral distribution of photosynthesis and the absorption spectrum. The investigator concluded from this that *Bact. photometricum* is capable of photosynthesis. In later years the investigations of VAN NIEL (25) have fully confirmed this in the case of purple bacteria in general.

The behaviour of *Bact. photometricum* in a spectrum was compared with the behaviour of the ciliate *Paramaecium bursaria* in which endozootic Chlorella's occur. Under anaerobic conditions this organism aggregates in a spectrum on the spots of maximum absorption of chlorophyll, *i.e.* where photosynthesis is most intensive. As *Bact. photometricum* showed a similar behaviour, ENGELMANN concluded that phototaxis in *Bact. photometricum* is also based upon a chemotaxis for oxygen. As will be described later on, the results obtained in the present investigation suggest a similar interpretation, in which, however, oxygen plays no part.

Next to the experiments of ENGELMANN, the work of BUDER (3) should be mentioned. BUDER succeeded in splitting up the region of aggregation in the blue-green, established by ENGELMANN, into three very weak, it is true, but nevertheless rather distinct regions at about 5300, 4900, and 4700 Å. The region of aggregation in the infra-red, the yellow, and the violet parts of the spectrum, as indicated by ENGELMANN, were also observed by BUDER. Comparison of absorption spectra of suspensions of living bacteria convinced BUDER of the activity of the complete pigment complex (consisting of bacteriochlorophyll and carotenoids) in relation to the

perception of the light. Though this conception follows not coercively from the data stated by BUDER (cf. VAN NIEL (27)), it was generally excepted afterwards (FRENCH (14), VAN NIEL (27)). We will learn presently that this conception does not hold accurately.

§ 2. STATEMENT OF THE PROBLEM.

From FRENCH's (14) measurements of the rate of photosynthesis as a function of wave-length in *Rhodospirillum rubrum*, it was established that light absorbed by the carotenoids, consisting for the greater part of spirilloxanthin (VAN NIEL and SMITH (26), POLGAR, VAN NIEL and ZECHMEISTER (30)) played no part in photosynthesis. He explained the accumulation of the bacteria in regions of bacteriochlorophyll by assuming a chemotaxis brought about by a gradient in concentration of carbon dioxide. This concentration gradient would be due to an unequal consumption of carbon dioxide in parts of the spectrum of different efficiency in relation to photosynthesis.

The aggregation of the bacteria in the places of lowest concentration of carbon dioxide can only be explained by accepting a flight reaction caused by a high concentration of this gas. Since, however, carbon dioxide acts as a substrate in photosynthesis, or in any case has a favourable effect on many metabolic processes (KLUYVER (18)), this conception does not seem probable. This conclusion is strengthened by the fact that the experiments of ENGELMANN and of BUDER yield no evidence in favour of the assumption that the physiologically favourable limits of concentration of this substance have been surpassed.

Instead of accepting the explanation of FRENCH, it seems more reasonable to consider a possible participation of the carotenoids in photosynthesis to such a limited extent that it has come within the limits of error of the measurements made by FRENCH. The fact that aggregation in the blue and green is much less distinct than in the yellow and infra-red, notwithstanding the strong absorption of light in these regions, is an indication in favour of this supposition.

We decided, therefore, to investigate this hypothesis by means of more exact methods, and have tried to furnish experimental proof for this conception.

§ 3. CHARACTERISTICS OF THE ORGANISM INVESTIGATED.

It appears from literature that in various organisms phototaxis

is often influenced by external or perhaps internal factors which apparently are difficult to control (STRASBURGER (32), FRANCÉ (13), MOLISCH (23), OLTMANNS (28), LUNTZ (19)). The variability in the behaviour of phototactic organisms may even be so strong that phototactic phenomena are completely lost because of some inscrutable reason.

Therefore, it appears rather essential to choose an organism which may be expected to be only slightly affected by differences in external factors, such as *e.g.* oxygen pressure, composition of medium, etc. Among the purple bacteria, such an organism is found in *Rhodospirillum rubrum*. As appeared from a preliminary microscopic examination of some strains of *Rhodospirillum rubrum*, originally isolated by VAN NIEL (27), the most suitable strain, No 4, indeed showed very regular phototactic reactions on a sudden decrease of the light intensity. MOLISCH (23) seems to have had the same experience with a strain of this species.

None of the spirilla responded to a sudden increase of the light intensity, nor did any of them respond to a decrease, if it were sufficiently gradual. However, merely shading the mirror of the microscope, or placing a Schott filter BG 17, which absorbs the phototactically highly active infra-red radiation, in the light-path caused all bacteria to reverse their direction of motion. This reversal was a very fascinating spectacle. The spirilla slackened their speed, stopped, and then moved back into exactly the opposite direction, apparently by reversing the direction of the beating of their flagella. The whole phenomenon resembled a shunting railway engine.

The spirilla, in crossing a boundary from light to darkness, in most cases penetrate into the dark for about half their length before moving backwards. When the contrast between light and dark is decreased, as *e.g.* in the case of weak light compared with a stronger illumination, the shock reaction is delayed, and a deeper penetration into the field of the lowest intensity occurs. It was found that the spirilla were extremely sensitive to contrasts; a difference of light intensity of about 7 % in some cases still just causing shock reactions.

The cultures of spirilla had been grown for about 48 hours under anaerobic conditions in a light-cabinet of 25—30° C. in a liquid medium with 1 % peptone Poulenc and ½ % NaCl.

§ 4. Determination of the action spectrum of phototaxis in *Rhodospirillum rubrum*.

Since the experiments of Engelmann and of Buder are of a qualitative nature, it is impossible to derive the action spectrum of phototaxis from their data. For the purpose of obtaining such an action spectrum, we employed, following a suggestion of Prof. Milatz, the quantitative principle of a compensation method.

On a slide under the microscope two small light fields were projected adjacent to each other with a sharp boundary between. The illumination of one of the fields could be varied in intensity as well as in wave-length, comparable with the situation in a photometer according to Lummer-Brodhun in which one of the fields is also variable in intensity. For this reason the set-up in which the principle of compensation was used, was named ,,the bacteriophotometer''. The following explanation will show what may be measured with the bacteriophotometer.

When the light-sensitive spirilla are moving in the field of the microscope, and both small fields are illuminated *e.g.* by white light, the spirilla moving under the cover glass will gradually accumulate in the field of the highest intensity. If both fields are illuminated by light of the same intensity and spectral composition, the bacteria will be homogeneously distributed over the two fields. When the light in the two fields possesses unequal spectral composition, but equal intensity, the bacteria will accumulate in the field whose light is photobiologically most active. For instance, if one of the fields is illuminated with green light, and the other one with red light of the same intensity — expressed in quanta/cm² sec. [1]) — all bacteria will collect in the green field, green light possessing a higher activity in phototaxis than red. By increasing the intensity of the red field, it is possible to obtain again a homogeneous distribution of the bacteria over both fields, in which case the bacteriophotometer is ''adjusted to equivalence''.

In such a case the fields are photobiologically equivalent, and on passage of the boundary no shock reactions of the spirilla are observed. If, *e.g.* the intensity of the red field was I_{red} quanta/cm²

[1]) Assuming Einstein-Warburg's law (34) to be valid for the light absorption process initiating the chain of events leading to a phototactic response; the activity of the light is proportional to the number of quanta absorbed by the spirilla. Hence, the intensity of the light must be expressed in quanta/cm² sec.

sec., and that of the green one I_{green} quanta/cm² sec., this means that I_{red} quanta of red light are photobiologically equivalent to I_{green} quanta of green light. We shall now define the ratio $\frac{I_{green}}{I_{red}}$ as the p h o t o t a c t i c a c t i o n of red light with respect to green. Substituting the red light by light of other wave-lengths, and again adjusting to equivalence with green, the ratio $\frac{I_{green}}{I_{\lambda}} = W_{\lambda}$ can be determined for each wave-length λ. Plotting the values determined in a diagram as a function of wave-length, we obtain a curve which, as will appear presently (cf. § 7), represents t h e a c t i o n s p e c t r u m (cf. fig. 1).

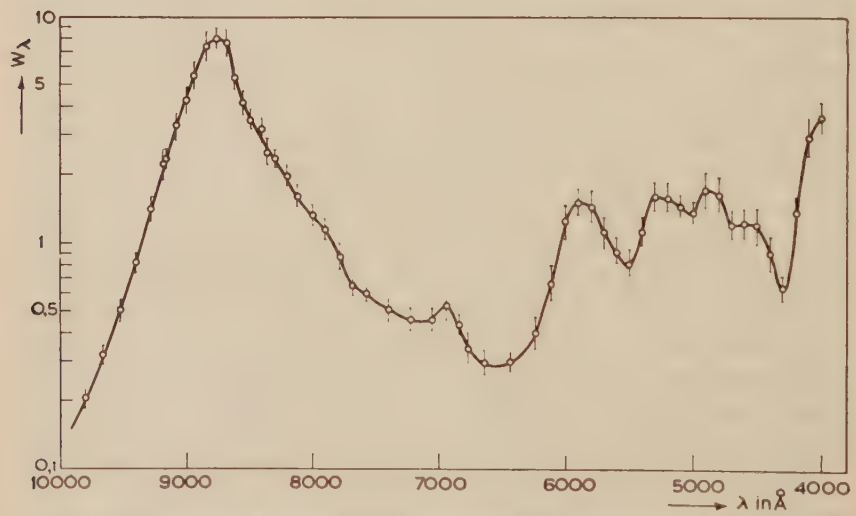


Fig. 1. The action spectrum of phototaxis in *Rhodospirillum rubrum*.

§ 5. Experimental set-up of the bacteriophotometer.

The action spectrum of phototaxis was measured with the help of the arrangement shown in the scheme of fig. 2.

We intented to obtain two homogeneously illuminated fields under the microscope. As the fields had to be illuminated homogeneously (variation limit 10 %), the size of each of the fields was limited, each of the rectangularly shaped fields being only 0.2 mm long and 0.15 mm wide.

Both fields at a'—b' (fig. 2) are focussed in the plane of the slide by the condenser C, and correspond to the field a—b in the double prism P. The part a—c of the diagonal plane between the right-

angled prisms in P reflects the light, whereas the part c—b transmits it. The prisms are partially cemented by a very thin layer of Canada balsam. In this way it is possible to obtain a very sharp boundary between both fields. This is necessary in order to prevent the bacteria from perceiving the boundary as a range of different intensity which might produce shock reactions, even when the illumination of both fields is equal. The light source I_1, a motor-car lamp (Philips 6V; 6A), illuminates the right field c—d. The source of light I_2 is a so-called projector lamp (Philips 6V; 7A, with mirror) the upright incandescent spiral of which is focussed upon the slit S_1 by the lens L_2, illuminating the left field a—c via the mono-

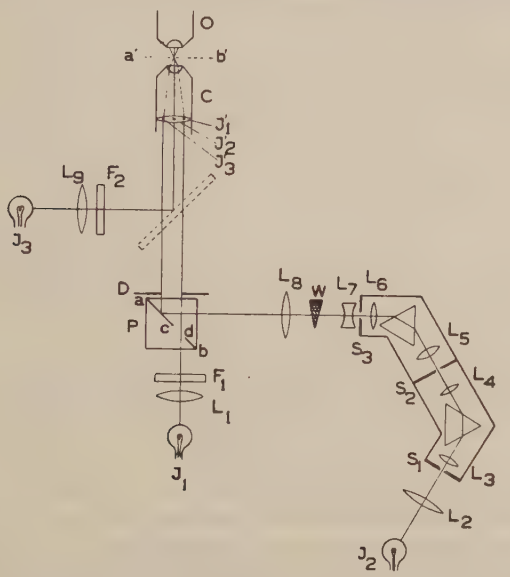


Fig. 2. Schematic diagram of the set-up used in measuring the action spectrum.

chromator and the weakener W. With a view to the required constancy of the fields at a—b and a'—b' both lamps are fed by the same large storage battery.

The monochromator according to VAN CITTERT (4) is adjusted in such a way that the loss of light is as small as possible. By adjusting the middle slit S_2 the monochromator can be made to transmit light of every desired wave-length. In order to get a homogeneously illuminated field in the double prism P at a—b, and on the slide at a'—b', the concave lens L_7 is mounted immediately in front of the end slit S_3, so that the plane of the homogeneously illuminated lens L_6 is focussed in the double prism upon the plane through a—b. The light beam, at the same time passes the lens L_8 and the adjustable logarithmic weakener W. The

image of the slit S_3 is projected by the lens L_8 via the reflecting part of P, in the plane of the large lens of the condenser C. As in the case in which the light comes from the lens L_1 which is placed as close as possible to the double prism P, the light from the slit S_3 is thus also focussed in a homogeneously illuminated field at a'—b'.

Finally, the light source I_3, for which a 12 Volt motor-car lamp was used, illuminates the whole microscopic field with a weak yellow-green light. This illumination was used in order to be able to detect the bacteria also, when working with infra-red radiation. A beam from this source passes the lens L_9, then the set of filters F_2, being identical with F_1 (Schott filters $VG3_{1mm} + 2 \times OG2_{2mm}$ + 1 cm 16 % $CuSO_4$ solution, the transmission-spectrum of this set shows one band at 5600 Å), and is reflected by a glass plate which acts as a semi-transparent mirror. By this system a strongly weakened image of the incandescent spiral is focussed on the condenser C. The spiral of I_1 is focussed in such a way that its image crosses that of the slit S_3 at right angles. The image of the incandescent spiral of I_3 is projected over the intersection of the images of I_1 and S_3. This intersection is projected exactly in the opening of the diaphragm of the condenser C. The aperture of the diaphragm is about 2.5 mm. A wider aperture has an unfavourable effect, probably due to reflections in the slide. This sets an upper limit to the intensity of the fields.

As it was necessary to maintain a sharp boundary between the fields, even when working with strongly different wave-lengths, the normal condenser with its great chromatic aberration was replaced by an achromatic Leitz No 4 objective of about the same focus.

The procedure of the measurement was as follows. The yellow-green light transmitted by the system of filters F_1, illuminating one of the two fields, was used as a constant light, with which the light from I_2, adjustable in intensity by the logarithmic weakener W and in wave-length by S_2, was compared. Then, for each wave-length the position of the weakener W was noted, at which a shock reaction could just be perceived in some spirilla, which were evidently more sensitive to contrasts than the other, when they crossed the boundary. In this way for each wave-length two positions of the weakener were found, corresponding with two light intensities. Between these positions no shock reaction of the bacteria occurred when they passed the boundary.

The measurement of the actual light intensities was carried out with a micro-vacuum-thermo-element which was especially constructed for measurements under the microscope, and was connected with a very sensitive Zernike galvanometer.

The receiver of the thermo-couple according to Moll (cf. 29) consisted of a small band of rolled out ,,thermo-plate'' (29) of only 50 μ width and 1 μ thickness. This small band was stretched between the copper leads of a lamp base, blackened by means of

a thin layer of soot and finally sealed into a T-shaped glass tube which was supplied with two plan-parallel windows (fig. 3). The thermo-element was evacuated by means of a double-stage mercury diffusion pomp, during which the element was heated at 150° C. Finally, the pressure of the mercury vapour was diminished during pumping by a treatment with liquid air. After this procedure the sensitivity of the element had increased about 90-fold. The glass-covered system (fig. 3) was enclosed in a case of brass with two round openings for the windows. During the intensity measurement the beam-shaped brass case was clamped upon the object-table of a Zeiss miscroscope, provided with a mechanical stage. Then at a small magnification the most sensitive spot of the small receiver, the welding-point, was brought into a field close to the boundary.

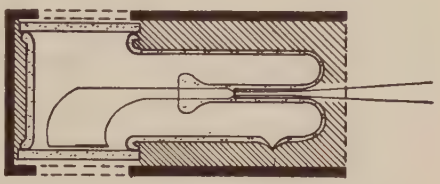


Fig. 3. Cross section of the micro-vacuum-thermo-element.

It must be remarked that the sensitivity of the absolutely calibrated thermo-element does not depend on the wave-length if the intensity of the light is expressed in ergs/cm² sec. Since it was necessary to correlate the phototactic action with the light intensity expressed in quanta/cm² sec., the light intensities read in ergs/cm² sec. were calculated into quanta/cm² sec.

The action spectrum was obtained as follows. Both limit values of the intensity, between which the spirilla did not show any shock reaction, were determined for each wave-length. These intensities, expressed in quanta/cm² sec., were divided by the intensity in quanta/cm² sec. of the yellow-green light in the other field, and the log-reciprocal values of the proportions were plotted versus the wave-length.

The action spectrum of fig. 1 shows that the region of maximum phototactic action are found at about 8800, 6900, 5900, 5250, 4900, 4600, and at 4000 Å. The present results are in good agreement with the data given by ENGELMANN (8, 10, 11,12) and by BUDER(3). The small maximum at 6900 Å has not been observed as yet.

§ 6. COMPARISON OF THE ACTION SPECTRUM WITH THE ABSORPTION SPECTRUM OF THE BACTERIAL PIGMENTS.

Since only light which has been absorbed by the pigment complex involved in phototaxis is active (GROTTHUS-DRAPER's law), we may

consider the absorption spectrum of the active agent to be reproduced in the action spectrum. A mathematical derivation (which will be omitted here; compare, however, (20, 22)), shows that the action spectrum and the absorption spectrum of the light sensitive substance are identical provided the absorption of light by the bacteria is small, and EINSTEIN-WARBURG's law (34) is valid. As the phototactic response of one bacterium served as an „indicator" in the adjustment of the bacteriophotometer to equivalence, the individual bacteria appearing absolutely colourless and transparent in the microscope, and, secondly, EINSTEIN-WARBURG's law must assumed to be valid (§ 4), we may accept the conditions required as being fulfilled.

In the literature the prevalent opinion regarding phototaxis of purple bacteria considers all pigments to be involved in phototaxis (ENGELMANN (8, 10, 11, 12), BÜDER (3), VAN NIEL (27)). However, as already remarked in the introduction, this conception is open to argument. Therefore, a more thorough study of this subject seemed desirable. To this end the total pigment system was isolated from the bacteria, and its components were analyzed.

The total pigment system was isolated as follows. The bacteria, after having been centrifuged, were ground in an agate mortar after the addition of some drops of water and a relatively large quantity of fine carborundum powder (KATZ and WASSINK (15)). After adding some water and centrifuging again for half an hour a transparent pinkish-coloured liquid was obtained. The absorption spectrum of the pigment complex (fig. 4) shows maxima at 8770, 8000, 7000, 5900, 5500, 5100, about 4800 Å, and in the violet.

When we compare the maxima of the action spectrum (fig. 1) at about 8800, 6900, 5900, 5250, 4900, 4600, and at about 4000 Å with this absorption spectrum (fig. 4), it appears that the maxima at 5250, 4900 and at about 4600 Å do not correspond with those of the absorption spectrum of the colloidal extract which contains the total pigment complex of the cells. This is especially evident in considering a sharp minimum at 5500 Å in the action spectrum which exactly coincides with an absorption maximum. That the action spectrum does not fully agree with the absorption spectrum is demonstrated all the more by the fact that the absorption of bacteriochlorophyll at 5900 Å (fig. 4) is smaller than that in the three adjacent maxima due to

carotenoids, contrary to what holds true for the maxima of the action spectrum in the corresponding regions.

The differences between absorption and action spectra being established beyond doubt, an attempt was made to isolate the pigments in question.

It follows from the above that not all pigments present in the bacteria are involved in the absorption of phototactically active light. It might be supposed that only the light absorbed by bacteriochlorophyll is active in initiating phototaxis. If true, the low maxima of action in the green and in the blue might represent

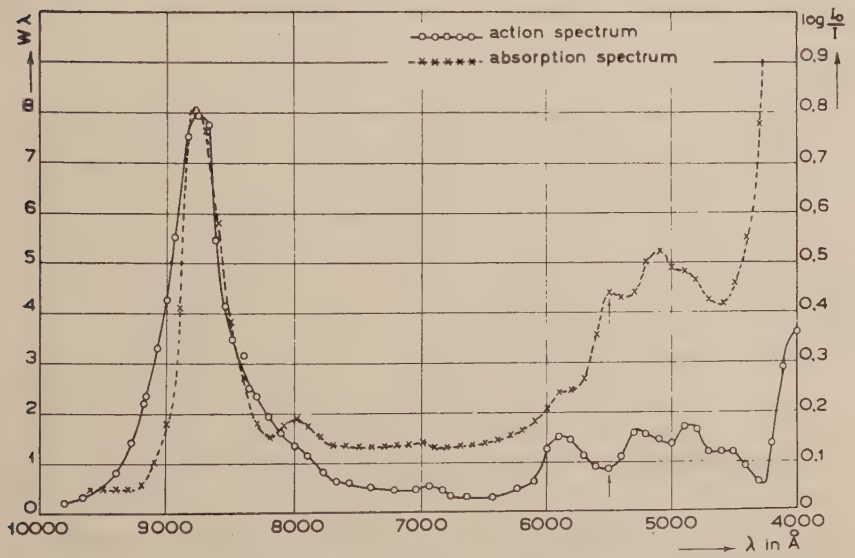


Fig. 4. The action spectrum of phototaxis (with a linear ordinate), compared with the absorption spectrum of the total pigment-complex of the cells.

small maxima in the absorption spectrum of this pigment. However, in the absorption spectrum of the living cells these maxima might be completely masked by the high absorption of the carotenoids in these regions, and consequently in this case would escape detection. If the light absorbed by carotenoids should not be phototactically active, this would give indirect support to the views defended by FRENCH (14) (cf. § 2). The absorption spectrum of bacteriochlorophyll given by VERMEULEN, WASSINK and REMAN (33), showing some maxima in the green and the blue, does not constitute an argument against FRENCH, since the former investigators have

employed a non-purified methanolic extract. Thus the possibility exists that the maxima in the green and in the blue may have been due to contamination of the bacteriochlorophyll by carotenoids. For this reason it was decided to attempt the preparation of pure bacteriochlorophyll.

Dr. WASSINK kindly drew our attention to the protecting action of an active reducing substance such as hydrogen sulphide on bacteriochlorophyll which in solution is very photolabile. The preparation of the solution of bacteriochlorophyll was carried out as follows. The centrifuged bacteria were extracted with absolute methanol which had been saturated beforehand with hydrogen sulphide. The deeply blue-green solution obtained in this way was

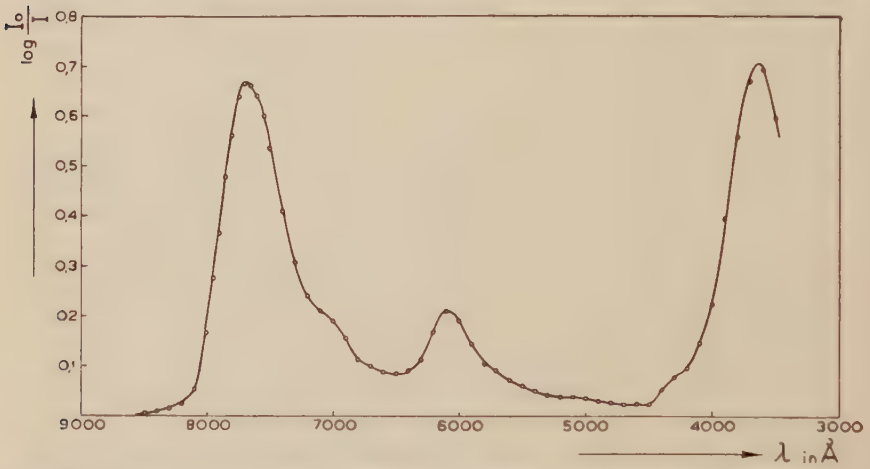


Fig. 5. The absorption spectrum of pure bacteriochlorophyll in methanol.

then mixed with an equal volume of H_2S-saturated petrol ether (b.p. 60—80° C.), yielding a homogeneous mixture of both liquids. The methanol was carefully washed out by means of water, and the petrol ether solution dried overnight with anhydrous sodium sulphate in an atmosphere of H_2S. Subsequently the solution was objected to chromatography in a column of BROCKMANN'S aluminium oxide. Development of the column by means of a mixture of petrol ether and 20 % thiophene-free benzene yielded a diffuse grey zone at the top of the column, and underneath a very intensely blue-green ring with a thin red ring, apparently consisting of carotenoids, some mm below it. After the blue-green ring had been cut out very carefully from the column, and the carotenoid-free

material eluted in methanol saturated with H_2S, the whole procedure was repeated. After development of this second column, the grey coloured zone was practically absent, while no ring of carotenoids could be detected. After eluting the blue-green ring in H_2S-saturated methanol, the absorption spectrum represented in fig. 5 was obtained. We consider this to be the absorption spectrum of pure, unchanged bacteriochlorophyll.

This absorption spectrum shows one principal maximum at 7700 Å (in the living bacteria this maximum is shifted to 8800 Å!), another at 3650 Å, and a lower maximum at 6070 Å, whereas in the region of 5500—4500 Å a broad minimum exists. Therefore, it is evident that bacteriochlorophyll is not responsible for all maxima in the action spectrum. This leads to the assumption that the three maxima of the action spectrum in the green and blue are identical with the absorption maxima of one or more of the carotenoids (KARRER and SOLMSSEN (16, 17)). Therefore, it was decided to isolate also the carotenoids present in *Rhodospirillum rubrum*.

The residue of the methanolic extract was extracted with acetone. The red solution of carotenoids obtained in this way was mixed with petrol ether. Next the acetone was removed by repeated shaking with water. Then the petrol ether solution was dried with anhydrous sodium sulphate and chromatographed in a column of Al_2O_3. To prevent destruction of the pigments the whole procedure was performed in weak (artificial) light. By washing the column with petrol ether containing 20 % thiophene-free benzene, and subsequently developing with dry benzene, three isolated bands and one ring were obtained. The upper and lower bands showed about the same bright-red colour, whereas the middle band, being by far the widest one, had a beautiful dark-red colour and obviously contained the main pigment spirilloxanthin. Each band was subsequently cut out from the column, dried in the air and the middle portion treated with methanol. Since, according to VAN NIEL (27) the location of the absorption bands of the isolated carotenoids most nearly approaches that of the bands of the pigments in the cell when dissolved in benzene, the pigments dissolved in methanol were transferred to this solvent by careful shaking, and subsequently dried with sodium sulphate. Fig. 6 shows the absorption spectra of the pigments in benzene, obtained in this way.

As is evident from a comparison of fig. 6 with fig. 1, the carotenoid

showing absorption maxima at 5430, 5070, and about 4800 Å can certainly not be involved in phototaxis. This is very remarkable, since in the spirilla this carotenoid prevails strongly. According to the wave-lengths corresponding to the absorption maxima this carotenoid is in all probability identical with spirilloxanthin, likewise isolated from *Rhodospirillum rubrum* by VAN NIEL and SMITH (26), or with rhodoviolascein (KARRER and SOLMSSEN (16, 17), or with α-bacteriopurpurin (MOLISCH (23)).

The unidentified carotenoid with absorption maxima in benzene at 5170, 4840, and 4550 Å can also not be involved in phototaxis.)

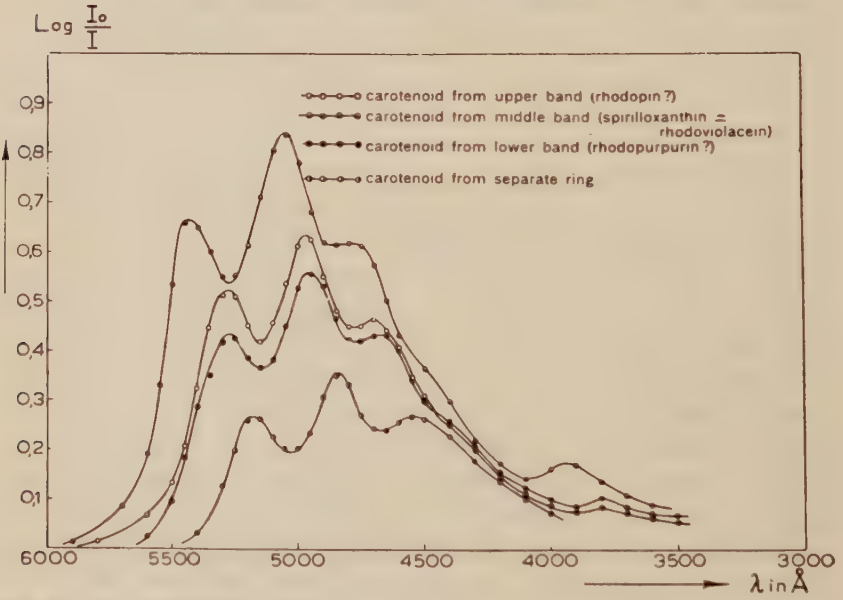


Fig. 6. The absorption spectrum of the isolated carotenoids in benzene.

However, the carotenoids with absorption maxima respectively at 5280, 4970, and 4700 Å, and at 5260, 4950, and 4670 Å may indeed be involved in the absorption of light active in phototaxis; the maxima of phototactic action at about 5250, 4900, and 4600 Å being in good agreement with the location of the absorption maxima of the carotenoids mentioned. From the data communicated by MOLISCH (23) and by KARRER and SOLMSSEN (16, 17), it may be concluded that these carotenoids are identical with the pigments of the β-bacteriopurpurin group (MOLISCH (23)), which were named rhodopin and rhodopurpurin by KARRER and SOLMSSEN.

Thus it is almost certain that, in addition to bacteriochlorophyll, (a) carotenoid(s) less abundant than spirilloxanthin, and similar to rhodopin and/or rhodopurpurin is responsible for the absorption of the phototactically active light.

§ 7 The relation between phototaxis and photosynthesis in *Rhodospirillum rubrum*.

The existence of a correlation between phototaxis and photosynthesis follows already from the fact that the principal assimilatory pigment, bacteriochlorophyll, is also involved in phototaxis. Further proof for this relation was given by the following experiments.

It was found that, beyond a certain limit, the contrast-sensitivity of the spirilla gradually decreased with increasing intensity. As this phenomenon appeared at light intensities of the same order of magnitude as the intensities at which light saturation of photosynthesis begins, it was regarded as being connected with the existence of limiting factors in photosynthesis. It was decided to investigate this phenomenon more in detail. Since it was necessary to extend the determinations of the contrast-sensitivity as a function of the light intensity over a wide range of light intensities, it was measured at various intensities of the green field against a field illuminated with infra-red radiation of 8800 Å; this radiation being very strongly absorbed by the bacteria and being present in abundance in incandescent light. So it was possible to apply intensities which are very high also in a physiological sense.

The different intensities of the green light were obtained by applying several combinations of Schott-filters in F_1 (fig. 2); F_2 being used in this experiments with only one OG1 filter.

In the following table the result of two measurements, performed at an interval of some days, is given.

The following data may serve as a further explanation of those represented in this table. The figures in column V were obtained by dividing the intensities (in ergs/cm² sec.) of the green field by the limit values of the intensity of the infra-red one. As in this case the intensities are not expressed in quanta/cm² sec., the relative phototactic action of infra-red towards green (average 14.5) is higher than it follows from fig. 1, in which it is about 8.

Table.

I	II	III	IV	
Intensity in ergs/cm² sec. of the green field	Intensity in ergs/cm² sec. of the infra-red field at which a shock reaction on entering the green field just occurred	Intensity in ergs/cm² sec. of the infra-red field at witch a shock reaction on entering the own field began to occur	Contrast-sensitivity of the shock reaction	Phototactic action infra-red: green
I_{green}	I_1	I_2	$\frac{I_{green}}{14.5 \times I_1}$	$\frac{I_{green}}{\sqrt{I_1 . I_2}}$
45000	51000	2010	0.06	—
37000	22500	1950	0.11	—
31000	8100	1790	0.26	—
26800	3650	1560	0.51	—
18000	1460	915	0.75	—
9200	720	525	0.88	14.9
6700	560	425	0.83	13.7
4000	330	245	0.835	14.0
2600	194	148	0.925	15.5
1140	92	67	0.86	14.4
56000	69000	2850	0.055	—
40000	7100	2400	0.39	—
30500	3310	1820	0.63	—
24000	2410	1580	0.69	—
15500	1270	955	0.84	14.1
6900	535	402	0.89	14.8

The figures indicating the action of the infra-red with respect to the green proves to be constant over a wide range of intensities, without reference to the fluctuation due to experimental sources of error. These figures show the phototactic action to be independent of the absolute intensity of the green field. This means that if the intensity of the green field *e.g.* decreases to one-third of the original intensity, then, in order to obtain equivalence, the intensity of the adjustable field must also be diminished to one-third of the original value. As will appear presently, the phototactic action as measured with the bacteriophotometer proves also to be independent of the conditions of environment (such as concentration of carbon dioxide and temperature) within wide limits. These facts emphasize the curve given in fig. 1 as indeed representing the action spectrum. Apparently this spectrum is not influenced by the conditions of the experiment, but represents merely a characteristic of the bacteria.

Further, the following important conclusions may be drawn from the table. When we define the contrast-sensitivity as the ratio of the intensities in both fields (of equal wave-length, or calculated at equal wave-length), for which a shock reaction of the spirilla in crossing the boundary just occurs, this ratio appears to be constant up to a certain intensity of the green field. Obviously, the contrast-sensitivity (the reciprocal value of the threshold value) of the phototactic response is not determined by a constant difference in light intensity, but by a certain ratio: in other words, phototaxis in *Rhodospirillum rubrum* follows WEBER-FECHNER's law (cf. SCHRAMMECK (31)).

The table further shows that the contrast-sensitivity of the bacteria is strongly reduced at higher intensities, after having shown a constant and maximum value below an intensity of the green field of about 10000 ergs/cm² sec. in one case, and below about 20000 ergs/cm² sec. in the other case. As this reduction begins to appear in a range of intensities where saturation of photosynthesis may be expected, it is tempting to suggest that the reduction of the contrast-sensitivity is due to the attainment of light saturation of photosynthesis.

Light saturation in photosynthesis finds expression in the shape of a so-called BLACKMAN curve (1), *i.e.* the curve obtained when plotting the rate of photosynthesis versus the light intensity. Such a curve is characterized by a straight ascending part, where the rate of photosynthesis is proportional to the light intensity, and by a horizontally deflecting part. Instead of plotting the velocity of photosynthesis against the light intensity, we can also map out the change in the rate of photosynthesis against the light intensity. At moderate intensities the change in the rate of photosynthesis will have a constant and maximum value which will decrease with rising intensities to zero as soon as the BLACKMAN curve has become horizontal.

When gathering the figures of column IV (cf. table) concerning the contrast-sensitivity as dependent on light intensity, in a graph, we get curves with a horizontal part at moderate intensities, and a part declining to zero at higher intensities (fig. 7). Apparently we meet with curves which are equally shaped as the curves obtained by plotting the change in the rate of photosynthesis versus

light intensity. This implies, evidently, that the contrast-sensitivity in *Rhodospirillum rubrum* is governed by the change in rate of photosynthesis.

Considering the experiments of ENGELMANN (8, 11, 12), in which it was established that only a sudden decrease in light intensity released a shock reaction (which was also observed in *Rhodospirillum rubrum* in the present investigation), this may be extended by the more general conception that a shock reaction is caused by a sudden drop in the rate of photosynthesis.

The arguments in favour of this conception may be summarized as follows.

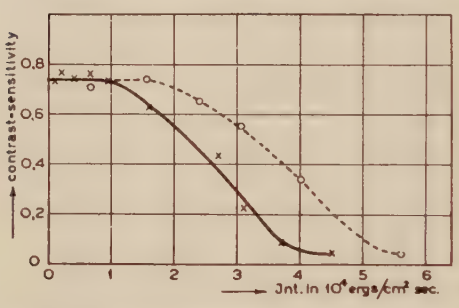


Fig. 7. The contrast-sensitivity of phototaxis in *Rhodospirillum rubrum* as a function of the light intensity.

1. Light absorbed by bacteriochlorophyll possesses a phototactic action.

2. The sensitivity of the spirilla to contrasts in dependence on light intensity is governed by the change in rate of photosynthesis as a function of light intensity.

3. The curves in fig. 7 show deflections at intensities which are generally characteristic of light saturation in photosynthesis (WASSINK, KATZ and DORRESTEIN (36)). This holds the more, because in *Rhodospirillum rubrum* the absorption of the infra-red is about eight times that of the sodium light employed by the latter investigators (cf. fig. 1).

4. The curves in fig. 7 show deflections in different ranges of intensity. Consequently the BLACKMAN curves, which can be derived from the curves in fig. 7 (cf. (20, 21)) also show deflections in different intensity regions. To all probability this is due to differences in temperature and/or CO_2 tension in the laboratory in the two days during which the experiments were performed. Since the rate of photosynthesis depends on temperature and CO_2 tension in a way similar to that of the curves deduced, this indicates them to be BLACKMAN curves indeed. Consequently this also constitutes a support of the conception in question.

Therefore, it is very likely that phototactic reactions in purple bacteria are induced by a sudden decrease of the rate of photosynthesis.

§ 8. Final considerations.

The following statement seems to be important, *viz.* that, since phototaxis in *Rhodospirillum rubrum* appears to be based upon a sudden decrease of photosynthesis, the conclusion must be drawn that the action spectrum of phototaxis at the same time represents the action spectrum of photosynthesis.

This implies that carotenoids are involved in photosynthesis. As in other organisms experience has taught that co-operation of carotenoids in photosynthesis usually is in the nature of a sensitization (24, 7, 38), it is thus probable that also in the case of *Rhodospirillum* the co-operation of the carotenoids in phototaxis and in photosynthesis has the character of a sensitization.

The present results evidently are at variance with those of French (14) who concluded from his experiments that light absorbed by the carotenoids is not active in photosynthesis. Evidently, the method used in the present investigation allows a much greater accuracy than the method used by French does.

The fact that in *Rhodospirillum rubrum* certain carotenoids too are involved in phototaxis and highly probably in photosynthesis which, moreover, according to the data furnished by Engelmann (10, 11, 12) and by Buder (3) must also be the case in other purple bacteria (*e.g. Bacterium photometricum, Thiospirillum, Chromatium*), might give cause for a generalization over the whole group of purple bacteria. However, it may be possible that in the various purple bacteria not always the same carotenoids are participating as in *Rhodospirillum rubrum.*

In addition to the possibility of different carotenoids being involved in phototaxis in various purple bacteria, it may be expected that different species will vary with regard to the distribution of phototactic sensitivity in the infra-red. For, whereas in *Rhodospirillum rubrum* the pigment-protein complex is characterized by one principal absorption maximum at about 8800 Å, all other purple bacteria investigated show two principal maxima of absorption in the infra-red which are most often located in the

neighbourhood of 8500 and 8000 Å (WASSINK, KATZ and DORRESTEIN (35), KATZ and WASSINK (15), WASSINK and MANTEN (37)). In this respect an examination of the behaviour of other purple bacteria in this region may be worthwhile.

About the same relation between phototaxis and photosynthesis as established in *Rhodospirillum rubrum* seems to exist in the green ciliate *Paramaecium bursaria* (ENGELMANN (9)). Under anaerobic conditions this organism accumulates in a spectrum in the region of 6500—7000 Å. This implies that in *Paramaecium bursaria* chlorophyll is involved in phototaxis. According to ENGELMANN, the nature of phototaxis in this ciliate would be a chemotaxis for oxygen evolved in photosynthesis. Also the underlying principle of phototaxis in purple bacteria was considered to be a chemotaxis for oxygen. In this connection it may be remarked that it is not necessary to accept any rôle of oxygen in phototaxis (or photosynthesis) of purple bacteria. It is also possible to consider phototaxis in *Rhodospirillum rubrum* as a chemotaxis for a certain product of photosynthesis found in the cell, for *Rhodospirillum* shows light saturation which, beyond a certain light intensity, finds its expression in the decrease of the contrast-sensitivity with increasing intensities. Evidently the phototactic phenomena are not correlated with the proportions of the concentrations of excited molecules of bacteriochlorophyll at two succeeding intervals, the relation between proportion in question and light intensity having not the shape of a BLACKMAN curve. However, it might be assumed that phototaxis in *Rhodospirillum* is associated with a change in the rate of production of a certain intermediate or even final product formed in the BLACKMAN process of photosynthesis.

Summary.

The action spectrum was determined of the phototactic reactions of the purple bacterium *Rhodospirillum rubrum* (Esmarch) Molisch. The method used was based upon the property of the spirilla of accumulating in the spots of highest intensity by executing shock reactions (avoiding reactions) at the boundary of surrounding spots of lower intensity.

Two differently coloured, adjacent small fields were projected in the slide with a dilute suspension of spirilla. One of the fields was kept constant, and acted as a field of comparison, whereas in

the other one the intensity, as well as the wave-length, of the light could be varied. The principle of the determination of the spectral sensitivity in the spirilla was based upon the adjustment of the ratio between the intensities of the two fields for different wave-lengths up to a degree just not perceived by the spirilla. The light intensities were measured with a micro-vacuum-thermo-element especially constructed for this purpose (cf. fig. 3). The action spectrum (cf. fig. 1) was obtained by dividing the intensity of the constant field by that of the variable field for several wave-lengths.

The action spectrum is not identical with the absorption spectrum of the pigment-protein complex in the living bacteria (cf. fig. 4). This is most evident from the minimum in the action spectrum at 5500 Å, at which wave-length a maximum is found in the absorption spectrum. It was found that the carotenoid spirilloxanthin which is found in abundance in the spirilla does not play a part in the absorption of light active in phototaxis. But this is the case with bacteriochlorophyll, and the carotenoids rhodopin and/or rhodopurpurin.

A solution of pure, unchanged bacteriochlorophyll has been prepared, and its absorption spectrum determined (fig. 5).

It is considered of special importance that it was possible to connect the decrease in contrast-sensitivity of the bacteria at high intensities with the attainment of light saturation in photosynthesis. From this, and from the fact that bacteriochlorophyll is involved in phototaxis, the conclusion was drawn that phototactic reactions of purple bacteria are induced by a sudden decrease in the rate of photosynthesis. This implies that the action spectrum of phototaxis in *Rhodospirillum rubrum* (cf. fig. 1) may be considered to be the same as the action spectrum of photosynthesis. Therefore also in purple bacteria light absorbed in certain carotenoids will be active in photosynthesis.

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(From the Bacteriological-Serological Laboratory of the University, Groningen).

AN INVESTIGATION ABOUT „ANTICOAGULASE", A FERMENT SAID TO BE PRODUCED BY STAPHYLOCOCCI

by

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I. Introduction.

In medical literature we find the following about the so-called ,,anticoagulase".

Neter (3) wrote that anticoagulase was produced by staphylococci in media containing carbohydrates. If anticoagulase is present, there is an absence of plasma-coagulation with either Ca-ions or staphylo-coagulase. It acts at once, so without any previous lag of action. Normal liquor and normal serum neutralize the action. Where there is coagulase or anticoagulase in an undiluted or in a slightly diluted culture medium the anticoagulase dominates, so that there is no coagulation; if the culture medium is further diluted the coagulase-action dominates, so that there is coagulation.

There are other bacteria (pneumococci, streptococci, coli bacteria etc.) that also excrete an anticoagulase in media containing carbohydrates. According to Neter the anticoagulase is possibly a derivative of the fermented carbohydrates. Finally he thinks of the possibility that the pH of the glucose-broth plays a part, although he does not think it very probable.

Birch-Hirschfeld (1) found anticoagulase in filtrates of staphylococcus cultures in ordinary broth and in filtrates which she prepared by cultivating staphylococci on cellophane-agar. She proved the presence of anticoagulase by letting the filtrate of the culture act on citrate-plasma for 12 hours at 37° C. in a ,,CO_2 atmosphere".

It is effective against the coagulation by thrombine, Ca-ions and staphylo-coagulase. The anticoagulase is resistent against a pH of 4.4 and 8.6 during 24 hours at 37° C., in contrast to coagulase. It is thermolabile and is absorbed by kaolin. BIRCH-HIRSCHFELD affirmed the discovery of NETER, that the anticoagulase-action takes place in the undiluted as well as in the weakly diluted filtrate, whilst, on the contrary, coagulase-action is produced in stronger dilutions. A filtrate containing anticoagulase would make fibrinogen and casein-solutions more stable against flocculation-substances (NaCl and acetic acid).

TILLETT (4) studied the appearance of anticoagulase in glucose broth cultures of haemolytic streptococci, *Streptococcus viridans* and pneumococci. He concluded that if the pH of the cultures was lower than 5, plasma coagulation was prevented. Acidified sterile broth was also able to prevent plasma coagulation.

TUNNICLIFF and HAMMOND (6) had noticed that smooth growing strains of *Streptococcus viridans* cultivated in glucose broth, prevented plasma coagulation, whilst rough growing strains did not show this phenomenon. On closer investigation as to the cause, they found that the pH of the glucose broth cultures of the smooth growing streptococci was much lower than that of the rough growing variety.

II. OWN INVESTIGATIONS.

With reference to the afore mentioned observations we investigated as to how far the prevention of plasma coagulation, which could be obtained by the aid of a glucose broth culture of a staphylococcus strain, could be ascribed to a decrease of the pH.

We repeated some of NETERS experiments and also found that there was an anticoagulase action in glucose broth cultures of staphylococci which could be stopped by serum and liquor.

After these experiments we continued our investigations, thinking that the pH might have something to do with the phenomenon. We tried to ascertain in how far an increasing pH did stimulate coagulation.

For our first experiment we used as coagulation factors:

1. A solution of 0.5 % $CaCl_2$ in 0.85 % NaCl.
2. A thrombine solution made according to the specifications of TILLETT and GARNER (5).

The experiments were carried out as follows. To 8 test tubes,

each containing 0.2 ml 2.75 °/$_{oo}$ K-oxalate human plasma we added successively:

a. Decreasing volumes of physiological salt solution. This was necessary because all experiments were carried out with increasing volumina of NaOH, resp. HCl with the object of getting a series with increasing, resp. decreasing pH. Therefore the plasma was diluted with decreasing quantities of physiological salt solution in order to make the final volume the same in all tubes.

b. To every tube 1 ml of culture filtrate to be investigated for anticoagulase activity; in the control tests, sterile broth or physiological salt solution was added.

c. Increasing volumes of NaOH (see f.i. Exp. A) resp. HCl (see f.i. Exp. B), both 0.02 n in physiological salt solution, so that a series of increasing, resp. decreasing pH was obtained. In consequence it was possible to determine whether plasma coagulation took place with different values of pH in each series.

d. In a first series of experiments (see Exp. A, B, C, D) 0.25 ml of a 0.5 % $CaCl_2$ solution was used as coagulation factor, and in the second series of the same experiments (see Exp. A′, B′, C′, D′) 0.1 ml of the prepared thrombine solution was added as a coagulation factor.

The results of the experiments were read after 30 minutes at 37° C. The pH of each tube was measured by means of a glass electrode and the intensity of the coagulation was estimated according to the following norms: ++ complete coagulation, + almost complete coagulation; ± moderate coagulation; —? a trace of coagulation; — no coagulation.

Experiment A.

In a preliminary test we determined that plasma coagulation by $CaCl_2$ was prevented by the addition of a filtrate from a glucose broth culture [1]) of a coagulase positive strain (M 13) of *Staphylococcus albus*. Now we ascertained whether by the addition of increasing quantities of 0.02 n NaOH to this mixture coagulation did appear by $CaCl_2$ and at which pH the change took place (Table I).

So it became apparent that after the addition of increasing quantities of 0.02 n NaOH, oxalate plasma, acidified with glucose broth filtrate, above a pH 5.78 was readily coagulated by 0.25 ml 0.5 % $CaCl_2$ solution.

[1]) The culture filtrate we used in Exp. A and the following experiments was obtained in the following way. A receptacle containing culture fluid of *Staphylococcus albus* M 13 grown during 4 × 24 hours at 37° C. was centrifuged for 15 minutes at a speed of 3200 rev. per minute. The clear fluid was filtered through a glass filter G 5/3 and then controlled for sterility.

Table I.

Tube No.	Plasma in ml	0.85 % NaCl in ml	glucose broth filtrate[1]) in ml	0.02 n NaOH in ml	0.5 % $CaCl_2$ in ml	coagulation	pH
1	0.2	1.6	1	—	0.25	—	5.03
2	,,	1.4	,,	0.2	,,	—	5.40
3	,,	1.2	,,	0.4	,,	±	5.78
4	,,	1.0	,,	0.6	,,	+	5.95
5	,,	0.8	,,	0.8	,,	++	6.22
6	,,	0.6	,,	1.0	,,	++	6.51
7	,,	0.4	,,	1.2	,,	++	6.78
8	,,	0.2	,,	1.4	,,	++	6.95
control 9	,,	1.6	,,	—	—	—	5.36
10	,,	1.6	1 ml sterile broth	—	—	—	7.48
11	,,	2.6	—	—	—	—	7.38

[1]) The pH of the glucose broth filtrate was 4.52.

Experiment B (see Table II).

We ascertained that plasma coagulation by $CaCl_2$ was not prevented by the addition of glucose-free broth culture of *Staphylococcus albus* strain M 13. We continued our experiments to see whether by addition of increasing quantities of 0.02 n HCl to this mixture, coagulation by $CaCl_2$ would now be prevented and if this were the case, at which pH this occurred.

The culture we used was again cultivated during 4 × 24 hours at 37° C. and then centrifuged and filtered. The pH of the filtrate was 6.35.

Exp. B showed that after the addition of increasing quantities of 0.02 n HCl, oxalate plasma, to which broth filtrate had been added, was no more clotted by $CaCl_2$ solution below pH 5.80.

Experiment C.

In this control test the broth filtrate of *Staphyloccus albus* strain M 13 from Exp. B was substituted by a sterile broth. We perceived that plasma coagulation by $CaCl_2$ was not prevented by the addition of sterile broth. It appeared that after the addition of

Table II.

Tube No.	Plasma in ml	0.85 % NaCl in ml	glucose broth filtrate in ml	0.02 n HCl in ml	0.5 % $CaCl_2$ in ml	coagulation	pH
1	0.2	1.6	1	—	0.25	++	6.60
2	,,	1.4	,,	0.2	,,	++	6.32
3	,,	1.2	,,	0.4	,,	±	5.80
4	,,	1.0	,,	0.6	,,	—	5.74
5	,,	0.8	,,	0.8	,,	—	5.49
6	,,	0.6	,,	1.0	,,	—	5.24
7	,,	0.4	,,	1.2	,,	—	5.08
8	,,	0.2	,,	1.4	,,	—	4.85
control 9	,,	1.6	,,	—	—	±	6.75 [1]
10	,,	1.6	1 ml sterile broth	—	—	—	7.36

[1]) It was seen that in the broth filtrate (tube No. 9) coagulase formed by the staphylococci was present, and that after 30 minutes at 37° C. there was a moderate coagulation of the plasma.

increasing quantities of 0.02 n HCl to this mixture, there was no coagulation by $CaCl_2$ solution below pH 5.91.

Experiment D.

In this control test without broth we now determined the pH below which diluted oxalate plasma, after the addition of increasing quantities of 0.02 n HCl, was not coagulated by a solution of $CaCl_2$. It appeared that after the addition of increasing quantities of 0.02 n HCl below pH 6.40 (because the buffering action of the broth is absent, this determination is very rough) oxalate plasma was no longer coagulated by a 0.5 % $CaCl_2$ solution.

In the same series of experiments, in which as a coagulation substance every time 0.1 ml of a thrombine solution was used (see page 89) the same results were obtained (Exp. A'-D').

Conclusions from experiments A-D and A'-D'.

a. The coagulation of plasma by a $CaCl_2$ solution and a thrombine solution does not take place any more below pH 5.8.

b. An ,,anticoagulase'' against $CaCl_2$ or thrombine is neither

shown in the glucose broth, nor in the broth culture of *Staphylococcus albus* strain M 13. The fact that the filtrate of a glucose broth culture prevents the coagulation of plasma by $CaCl_2$ solution or thrombine solution should be ascribed to the decrease of the pH below 5.8.

In order to make our experiments complete we extensively studied by which amount of acid, plasma coagulation by staphylocoagulase was prevented.

We prepared a fluid containing coagulase in either of the following ways:

a. We centrifuged a 4 × 24 hours old broth culture of the coagulase positive strain M 13 of *Staphylococcus albus*, and filtered the clear fluid through a glass filter G 5/3.

b. According to the method of GENGOU (2) by heating the upper fluid of a centrifuged culture during 90 minutes at 65° C.

The sterility of the coagulase-containing fluids, obtained by the methods *a* and *b* was controlled by repeatedly adding 1 ml to broth, which was examined for sterility during 3 × 24 hours.

Experiment E.

We investigated whether the addition of increasing quantities of 0.02 n HCl prevented the coagulation of oxalate plasma by staphylocoagulase prepared according to method *a*. After 4 hours at 37° C. the results were read as mentioned in Table III.

Table III.

Tube No.	Plasma in ml	0.85 % NaCl in ml	staphylo-coagulase M 13 in ml	0.02 n HCl in ml	coagu-lation	pH
1	0.2	1.6	1	—	++	7.20
2	,,	1.4	,,	0.2	+	6.71
3	,,	1.2	,,	0.4	+	6.42
4	,,	1.0	,,	0.6	±	5.93
5	,,	0.8	,,	0.8	—	5.70
6	,,	0.6	,,	1.0	—	5.21
7	,,	0.4	,,	1.2	—	5.01
8	,,	0.2	,,	1.4	—	4.85
control 9	,,	1.6	1 ml sterile broth	—	—	7.48

Experiment F.

VANBREUSEGHEM (7) pointed out that the staphylo-coagulase would for the greater part be destroyed by the acid formed in glucose broth, so that after neutralizing of a glucose broth culture, this would show no sign of coagulase action.

To ascertain whether the conclusion of VANBREUSEGHEM was correct, we investigated whether plasma coagulation by a filtrate of glucose broth culture M 13 would be made possible by the addition of increasing quantities of NaOH.

After 4 hours at 37° C. the results were read as mentioned in Table IV.

Table IV.

Tube No.	Plasma in ml	0.85 % NaCl in ml	glucose broth filtrate in ml	0.02 n NaOH in ml	coagulation	pH
1	0.2	1.6	1	—	—	5.08
2	,,	1.4	,,	0.2	—	5.24
3	,,	1.2	,,	0.4	—	5.49
4	,,	1.0	,,	0.6	—	6.0
5	,,	0.8	,,	0.8	±	6.45
6	,,	0.6	,,	1.0	+	6.75
7	,,	0.4	,,	1.2	+	6.82
8	,,	0.2	,,	1.4	—[1])	7.25
control 9	,,	1.6	1 ml sterile glucose broth	—	—	7.48

[1]) After 24 hours tube no. 8 was completely coagulated.

This experiment with fluid containing coagulase, prepared according to method *b* (page 92) gave exactly the same result.

Thus, it was proved that staphylo-coagulase was not, or merely very slightly destroyed by the acid formed in a glucose broth culture M 13. The addition of increasing quantities of 0.02 n NaOH restored the coagulation of oxalate plasma by staphylo-coagulase formed in a glucose broth culture.

Conclusion from experiments E and F.

a. Acid prevents coagulation of oxalate plasma by staphylo-coagulase below a pH of about 5.9.

b. An anticoagulase directed against staphylo-coagulase is not ascertained.

c. The staphylo-coagulase was not destroyed by acid; this is contrary to VANBREUSEGHEM's theory.

Corresponding results were obtained with fluids containing coagulase, prepared from cultures of *Staphylococcus aureus*, strains 5633/34 and 12996/97.

BIRCH-HIRSCHFELD reported the following facts about the ,,anticoagulase":

1. The ,,anticoagulase" is adsorbed by kaolin.
2. The ,,anticoagulase" partly disappears when heated.
3. The ,,anticoagulase" can withstand pH 4.4 and 8.5 for a period of 24 hours at 37° C.

Experiment G.

With reference to point 1 we made the following experiment. A filtrate from a 2 × 24 hours old glucose broth culture M 13 was divided into two parts. To the first portion we added 10 g of kaolin. The receptacle containing this mixture was first thoroughly shaken and then put into the refrigerator for 24 hours at + 4° C. The untreated portion was kept for control. The pH of the filtrate treated with kaolin was 5.0, that of the untreated portion was 4.72. We determined that:

a. The untreated filtrate with pH 4.72, as was to be expected from the above mentioned results, prevented the coagulation of a 2.75 °/$_{oo}$ K-oxalate plasma, diluted with physiological saline solution, by $CaCl_2$ and thrombine solution. Neither was coagulation caused by the staphylo-coagulase present in the filtrate (see Exp. F).

b. The filtrate treated with kaolin (pH 5.00) did n o t prevent coagulation of the 2.75 °/$_{oo}$ K-oxalate plasma, diluted with physiological saline solution, by thrombine and by $CaCl_2$ solution. The staphylo-coagulase formed in the filtrate did also coagulate the plasma.

This experiment confirmed the correctness of BIRCH-HIRSCHFELD's observations. She, however, attributed the fact, that after treatment with kaolin, the filtrate did not prevent plasma coagulation, to the destruction of a s p e c i f i c a n t i c o a g u l a s e f o r m e d b y t h e s t a p h y l o c o c c i.

However in view of our results it is possible that the increase of the pH from 4.72—5.00. by treatment with kaolin raises the pH of the whole mixture, which is influenced by the buffering action of the plasma, the dilution, etc. to such a degree that coagulation can take place again. The following experiment confirms this opinion (Table V).

Table V.

Tube No.	Plasma in ml	0.85 % NaCl in ml	glucose broth filtrate untreated in ml	0.5 % $CaCl_2$ in ml	Thrombine in ml	coagulation	pH
1	0.2	1.6	1	0.25		—	5.31
2	,,	,,	,,		0.1	—	5.38
3	,,	,,	,,			—	5.37

Tube No.	Plasma in ml	0.85 % NaCl in ml	glucose broth filtrate in ml treated with kaolin	0.5 % $CaCl_2$ in ml	Thrombine in ml	coagulation	pH
4	0.2	1.6	1	0.25		+	6.30
5	,,	,,	,,		0.1	+	6.45
6	,,	,,	,,			+	6.30
7	,,	,,	1 ml sterile glucose broth			—	7.20

The pH of the untreated filtrate was 4.66. When the filtrate was treated with kaolin the pH rose to 5.15.

The above experiment was repeated with a filtrate from a 3 × 24 hours old glucose broth culture of the coagulase positive strain 5633/34 of *Staphylococcus aureus*. These results corresponded with those obtained in Exp. G.

Conclusion from experiment G.

The neutralizing of the anticoagulating action of a glucose broth filtrate of a staphylococcus after treatment with kaolin must be attributed to the raising of the pH of the filtrate and not to the inactivation of the anticoagulase formed by the staphylococci.

As an experiment proved that kaolin did not give off any alkali to the physiological saline solution, we believe that the increase of pH by treatment with kaolin must be attributed to the adsorption of acid metabolic products of the staphylococci.

Summary.

2.75 °/$_{oo}$ K-oxalate human plasma was not coagulated below a pH 5.8—5.9, either by a $CaCl_2$ solution, or by staphylo-coagulase. The prevention of plasma coagulation by the filtrate of a glucose broth culture of staphylococci must be ascribed to the decrease of pH.

The experiments made by TILLETT (4) and TUNNICLIFF and HAMMOND (6) in studying the anticoagulation action of glucose broth cultures of streptococci are herewith confirmed. The neutralizing of the anticoagulating action of a filtrate of a glucose broth culture of staphylococci after treatment with kaolin must also be ascribed to an increase of pH caused by adsorption of acid metabolic products of the staphylococci.

Staphylo-coagulase is n o t destroyed at pH 4.5.

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ON THE PRODUCTION OF FOOD YEAST FROM PARTIALLY HYDROLYZED CEREAL STRAW

by

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1. Introduction.

Shortly after the german invasion of the Netherlands in 1940 the possibility was considered of a shortage of protein fodder for domestic animals and the idea occurred to produce food yeast from straw (the only abundant raw material available) through partial acid hydrolysis in a simple manner which could be carried out in existing equipment of strawboard factories without elaborate changes.

Financial support for an investigation of the possibility of such a production was sought and obtained from the Director-General of the Government Bureau for Food Supply. The funds were placed in the hands of a comittee of Prof. Dr. O. de Vries (chairman), Ir. L. H. de Langen and Ir. E. L. Ritman.

The following institutions cooperated in this investigation: the Microbiological Department of the Agricultural Experiment Station at Groningen, the Cooperative Strawboard Factory ,,De Eendracht'' at Appingedam, and the Netherlands Experiment Station for the Utilization of Straw at Groningen. The work was started at the last-mentioned institution with the development of a method for the partial hydrolysis of straw, while work on yeast production from the extracts obtained was soon started at the first-mentioned one; meanwhile a semi-technical installation was constructed at Appingedam, were the preparation of the extracts was taken over from the Straw Station. Later on the work

was concentrated at Appingedam, at first on a laboratory and afterwards on a semi-technical scale. Moreover, some special problems concerning the growth of the yeast in straw hydrolyzates were investigated at the Microbiological Department at Groningen. Throughout the work the Straw Station acted as an administrative and coordinative agency. Finally, an additional investigation concerning yeast growth in straw extracts and pure sugar solutions was carried out at the Straw Station.

With the exception of those of the last mentioned work, the results of these investigations were laid down in a report to the Director-General mentioned above.

The results of the present investigations did not lead to an industrial application chiefly because of the difficulty to find an outlet for the residue of the hydrolysis (amounting to 65-70% of the raw material); moreover, conditions during the german occupation did not favour new industrial developments. Publication of the results was prevented until now by war and post-war conditions. However, recent interest in food yeast production in various countries (e.g. British Empire (25-27), Spain (17) and U.S.A. (1, 8, 9, 10, 18, 19, 23, 24, and 29) has conveyed the impression that these results may still be useful to other workers in the field.

It is not intended to give an extensive review of the rather voluminous literature on production and properties of food yeast.[1]) References to the literature pertaining to the present investigations will be made in the following chapters. The second chapter deals with the preparation and the composition of the acid straw extracts. In the third chapter the laboratory culture experiments are discussed and in the fourth chapter those on a semi-technical scale. On a laboratory as well as on a semi-technical scale serious difficulties were encountered in the beginning which it took some months to overcome. Within the scope of this paper it is impossible to give an exposé of the course of these investigations, which were started with laboratory cultures on straw hydrolysates and glucose and were continued with cultures on these and other substrates (ethanol and acetic acid) and alternated with semi-technical cultures on the two first-mentioned ones. In chapter 3 the cultures on pure substances (ethanol, acetic acid and sugars) will be discussed

[1]) A fairly complete bibliography is given in (17).

first, followed by those on straw hydrolysates, with a short discussion of the causes of the initial difficulties.

Throughout the investigations a strain of *Torulopsis utilis* was used which was obtained from the yeast division of the „Centraal Bureau voor Schimmelcultures" at Delft. The work of FINK and his associates (2, 5, 11-16) has shown that this species can also use pentoses as a substrate, which sugars predominate in the partial hydrolysates employed.

2. PREPARATION AND COMPOSITION OF THE ACID HYDROLYSATES.

Extensive laboratory work led to the conclusion that the most appropriate conditions for the partial hydrolysis were heating of the chopped straw in an autoclave with the fourfold quantity of 1% sulfuric acid during one hour at 120-135° C. This yielded 20-25% of reducing sugar (calculated on oven-dry straw)[1]), and a residue of 65-70%. One half of the 10% loss consisted of inorganic constituents and the other half of organic matter, i.a. a lignin-like substance and acetic acid.

Meanwhile a 100 l globular rotary digester was installed at Appingedam. It consisted of two halves with flanges bolted together, the right half carrying a man-hole the cover of which was provided with a blow-off plug and a thermometer well. The digester was internally coated with an ebonite mixture.

On behalf of the special investigations carried out at Groningen the following extraction apparatus was constructed.

A 12 l round glass flask with rubber stopper and a glass tube reaching to the centre of the flask was fixed in an iron basket, which could rotate in a steam sterilizer. The basket was connected to an axis which protruded pressure-tight from the wall of the sterilizer and which was driven by an electromotor at a speed of 40-60 revolutions per minute. Care had to be taken to protect the manometer from ammonia vapour which corroded the thin copper spiral of the meter in a short time. In this way 1 kg straw could be treated with 4 l diluted acid (1%) yielding a solution which contained 2.1-2.3% reducing sugars, and 0.26-0.34% acetic acid. The extract was neutralized with chalk, filtered and the necessary nutrient salts added.

At Appingedam the extracts were prepared as follows. The

[1]) The reducing sugar was determined according to the LUFF method as decribed in (20).

digester was preheated with steam and subsequently filled with 10 kg of chopped straw and 40 l of boiling 1% sulfuric acid. After closing the man-hole the digester was rotated and heated by steam injection to the working temperature after which steam was blown off to atmospheric pressure in order to remove the occluded air. The digester was heated to the working temperature and kept on it for the prescribed time, after which the pressure was blown off again and the digester emptied in buckets. When the digester was operated for laboratory experiments a sufficient amount of extract could easily be drawn off from the residue; the separation of extract and residue for the semi-technical experiments will be described in chapter 4,b.

A number of experiments were made in order to determine the most favourable conditions of hydrolysis in the digester with the above-mentioned concentration and quantity of sulfuric acid. The maximum amount of reducing sugar was obtained after 1 hour at 3 atm. steam pressure (temp. 137-138°C), yielding about 20% of sugar calculated on oven-dry straw.

For the culture experiments the extracts were neutralized with $CaCO_3$ or $Ca(OH)_2$, filtered and diluted to a concentration of reducing sugars of about 2%. Some analytical data for a couple of extracts are given below.

Date of preparation	% of ash	% of reducing sugar	% of hexose[1])	% of pentose[1])	% of acetic acid[2])
1-27-43	0.78	2.11	0.32	1.79	0.25
4- 7-43	--	2.21	0.46	1.75	0.27

[1]) The pentose content was determined according to the method described in (21) p.54; the hexose content was calculated by subtracting the pentose figure from that of the reducing sugar (the reduction values of glucose and xylose being the same).

[2]) The acetic acid content was determined according to the method described in (22) p.35, with the difference that thrice 90 ml was distilled off and collected in an empty receiver. Titration was thus carried out in a distillate volume of 270 ml.

These data show that, besides pentose (practically xylose only), appreciable quantities of hexose (practically glucose only) and acetic acid occur.

3. Experiments on a laboratory scale.

a. Methods employed.

Culture Vessel.

An apparatus for growing fodder yeast on a laboratory scale should be equipped as follows: a capacity of 4-8 l, accomodating 2-4 l of medium and an equal volume of foam; an aerating device which produces a steady stream of very fine air bubbles; a glass and a calomel electrode to determine the pH of the medium; a device to regulate the pH by adding acid or alkali; a tube with a stopcock at the bottom to take samples; a device to measure the quantity of the air used for aeration (rotameter, gasmeter). The apparatus developed by the authors in analogy to the one used by Fink e.a. (6) consisted of a long glass tube (diam. 7.4 cm, length 85 cm); for aeration a porous stone, as used for the aeration of aquaria, was fixed in the bottom with a rubber stopper. The pH was controlled by means of a double set of electrical pipettes, one for adding 10% NH_4OH, the other for adding 20% HCl (fig. 1).

The design of the electrical pH control was as follows: the potential difference between glass electrode and calomel electrode was led through a shielded cable to the grid of an electrometer triode (Geco valve type T.). The resulting increasing or decreasing anode current activates a sensitive galvanometer, the mirror of which threw a light beam on one or the other of two photocells (Philips 3530). After amplification one or the other of two relais was energized causing a small motor to rotate the contact of a large, wire-wound potentiometer to the right or to the left. In this way the anode current was compensated after which the light beam rested between the photocells (system according to Wallace (30)).

The potentiometer shaft was connected with a cord to a small carrier, carrying a recording pen and a contact. In this way the place of the pen and the contact corresponded to a certain pH. For pH control two movable contacts could be fixed at places corresponding with the desired maximum and minimum pH. As soon as the contact on the carrier touched one of these contacts, one of the electromagnetic pipettes was opened and one ml of NH_4OH or of HCl was added to the wort, after which the pipette was refilled automatically. At the same time the

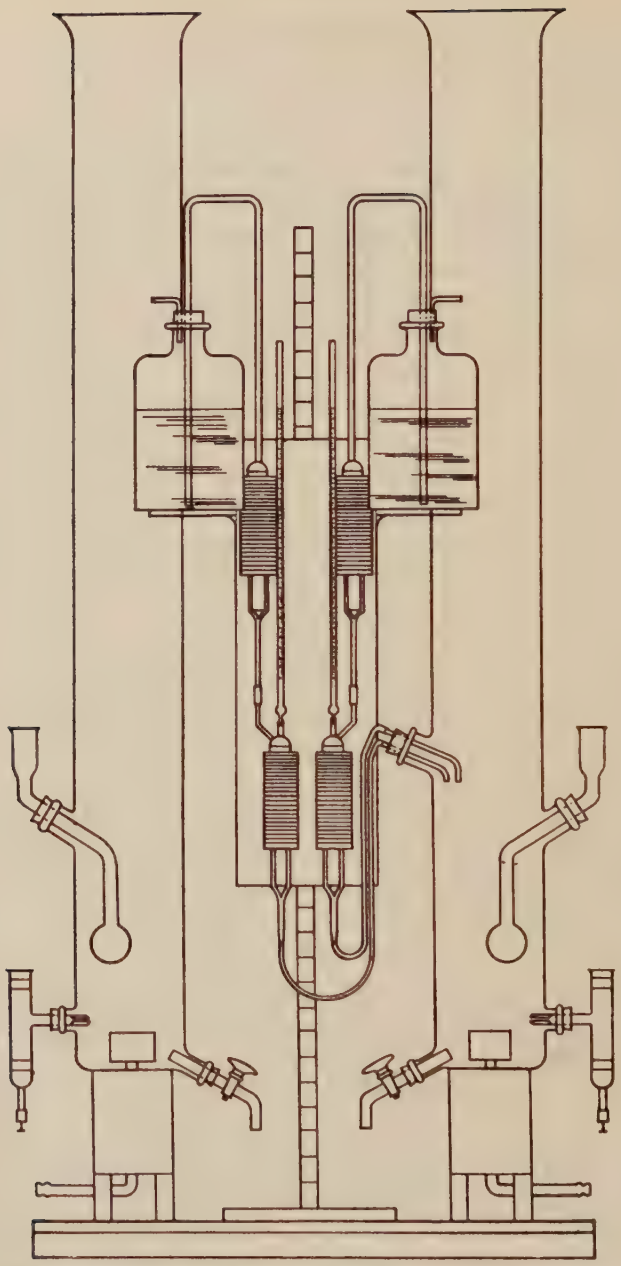


Fig. 1. Glass apparatus for growing food yeast on a laboratory scale. The pH is automatically regulated by means of a pair of electrically controlled pipettes, adding acid or alkali. Note the porous stone at the bottom and the glass and calomel electrodes for pH control and registration.

pH changes were recorded with the recording pen attached to the carrier.

Foam control.

Intensive aeration of the straw extracts produced a persistent foam, which was difficult to control. A quick rotating device, which constantly sprayed extract into the foam did not answer the expectations. For experiments on a laboratory scale surface-active substances proved to be most efficient means of foam control. Of these octadecyl alcohol had the advantage of being odourless and of not interfering with yeast production. With „Etingal", a product of the I. G. Farben-Industrie (a mixture of different alcohols) the foam disappeared instantaneously, but reappeared within a short time, so that it had to be applied rather often.

Yeast determination.

The yeast content of the medium was determined gravimetrically as well as with the aid of a nephelometer. In the first case an aliquot part of the medium was filtered through a Jena glass filter (1G 4) after a few drops of HCl 10% had been added to dissolve the phosphates, which contingently had been precipitated in the culture solution. Too much acid had to be avoided, as this reduced the dry weight of the yeast. The yeast was washed a few times with distilled water, dried at 105°C. and weighed. For tracing the propagation of the yeast during an experiment a nepholometer was constructed consisting of a glass cuvette, containing 50 ml of the diluted yeast suspension, which was illuminated from aside by a small electric lamp. The light reflected by the yeast cells fell on a selenium photronic cell and the resulting photocurrent was read by means of a micro-ampère meter. When the medium was sufficiently diluted (1 ml on 49 ml tap water) a fairly linear relation appeared to exist between the number of yeast cells and the photocurrent.

In the beginning the results were checked by gravimetric determination of the yeast.

b. Experiments with pure substances.

Throughout the investigation the following medium (indicated by Lechner (12)) was used, unless stated otherwise:

TABLE I.

Laboratory cultures on ethanol and acetic acid.

Date 1941	Medium	Inoculation	Duration h	Yeast production g/l	Substrate content % before	Substrate content % after	Yield % of substrate converted	Remarks
4/4 *a*	2.5 l 0.3 % ethanol with salts, 56 ml 96 % ethanol added afterwards	17.5 g from ethanol culture	9½	4.08	2.00	0.00	20	aeration *a* and *b* together 8.8-49.3 l/h. pH 1st. 6 h 5.0-6.0, afterwards 4.0-6.0.
4/4 *b*	as 4/4 *a*	as 4/4 *a*	9	4.56	2.00	0.00	23	pH 1st. 5 h 5.5.-6.0, afterwards 4.0-6.0.
4/17 *a*	as 4/4 *a*	as 4/4 *a*	9	7.60	2.00	0.00	38	aeration *a* and *b* together 14-81 l/h. pH 1st. 3 h 5.5-6.0, afterwards 4.5-6.0. 0.06 % acetic acid formed.
4/17 *b*	as 4/4 *a*	as 4/4 *a*	9	7.88	2.00	0.00	39	pH 1st. 4 h 5.5.-6.0, afterwards 4.3-6.0. 0.06 % acetic acid formed.
4/18 *a*	2.5 l 0.3 % acetic acid with salts, 29.0 ml 97 % acetic acid added afterwards	17.5 g from ethanol culture	14	5.12	1.46	0.00	35	aeration *a* and *b* together 18-104 - 85 l/h. pH about 4.5.
4/18 *b*	2.5 l 0.3 % acetic acid with salts, 41.5 ml 97 % acetic acid added afterwards	as 4/18 *a*	14½	6.44	2.00	0.09	34	pH about 5.7.
4/24 *a*	2.0 l 0.3 % acetic acid with salts, 28.1 ml 97 % acetic acid added afterwards	14 g from 4/18 *a*	10¾	4.88	1.77	0.00	28	aeration *a* and *b* together 20-200 l/h. pH about 4.5.
4/24 *b*	2.0 l 0.3 % acetic acid with salts, 33.0 ml 97 % acetic acid added afterwards	14 g from 4/18 *b*	11	7.52	2.02	0.00	37	pH about 5.7.

$(NH_4)_2HPO_4$	0.11 %
$(NH_4)_2SO_4$	0.2 %
K_2SO_4	0.06 %
$MgSO_4 . 7H_2O$	0.09 %

In the beginning of the investigation a few good results were obtained, both with a mineral glucose medium and with straw extracts, but these could not be reproduced at will. It was not until a gas flow meter was installed for measuring the amount of air supplied to the cultures that the principal cause of the many failures, *viz.* insufficient aeration, was detected. The influence of the aeration, also with a non-fermentable substrate, is clearly shown by a comparison of the cultures on ethanol on 4/4 and 4/17-41, resp. (see table I): the former giving a yield of dry yeast, calculated on substrate converted, of 20 and 23% with an aeration of 4.4-24.7 l/h per culture, and the latter of 38 and 39% with an aeration of 7.0-40.5 l/h per culture. The latter yields are well within the range found by FINK and KREBS (3, 4), the more so when it is borne in mind that no correction was made for evaporation of the ethanol from the medium. However, as the ethanol concentration was kept at or below 0.3%, the evaporation loss will have been small.[1])

The experiments with acetic acid in table I are of importance in a different respect. During November and December, 1940 and January 1941, the results with straw extracts became poorer and poorer and finally it appeared that the yeast had died off, in spite of its still turgescent appearance, the only remarkable feature being a strong vacuolisation. In the first acetic acid culture made (not mentioned in table I) the initial pH was about 4, which had also often been the case in the preceding straw extract cultures. It appeared that the yeast in this culture showed a great many vacuoles which had been absent in the inoculum (originating from a glucose culture). Moreover, the pH in this culture remained practically constant for $4^1/_2$ h, which showed that no acetic acid was converted. Conversion set in slowly after the pH had been brought to 5.0.

These phenomena indicate that even 0.3% acetic acid is quite

[1]) In tables I, II and IV the figures in column 6 for ethanol, acetate and glucose cultures designate the t o t a l quantity of substrate added, calculated as % of the f i n a l amount of medium.

toxic to *Torulopsis utilis* at a pH of about 4 and less so at a higher pH. Similar observations were already made in 1915 by HÄGGLUND (7) for *Saccharomyces cerevisiae.*

As the straw extracts usually contained 0.2-0.3% acetic acid, it was considered probable that the difficulties experienced during the above-mentioned period were at least partly due to a toxic effect of this acid in the extracts, especially when the initial pH was below 5.0. For this reason the pH in further straw extract cultures was maintained at 5.5-6.0 until the acetic acid had disappeared.

Since in the above-mentioned first acetic acid culture the air supply had not been controlled and thus might have been inadequate, some more cultures were made with an amply sufficient aeration. On 4/18 two cultures were started with yeast grown in ethanol, one being kept at a pH of 4.5 (*a*) and the other at 5.7 (*b*). As in the ethanol cultures, the substrate concentration was kept at about 0.3%. Both showed the same percentage yield, but the one at pH 4.5 converted the acetic acid at a distinctly lower rate than the one at pH 5.7 (mean conversion 1.04 and 1.32 g/lh resp.). Vacuoles were observed in the yeast crops of both cultures, but were more numerous in 4/18*a* than in 4/18*b*. The experiment was repeated on 4/24, using yeast from 4/18*a* as the inoculum for 4/24*a* and yeast from 4/18*b* for 4/24*b*. The latter culture showed about the same yield as the corresponding parent culture, whereas the yield of the former was distinctly lower than that of 4/24*b* (37 and 28% resp.) as well as than that of its parent culture (35%). Again the rate of conversion of the culture at pH 4.5 was lower than that of the culture at pH 5.7 (1.65 and 1.84 g/lh resp.), while the yeast in the former showed a stronger vacuolisation than that in the latter. A yield of 37% checks well with those obtained by FINK and KREBS (3, 4). Thus, these results constitute a strong indication for a cumulative adverse effect of acetic acid on *T. utilis* at a pH of 4.5 as compared with a pH of 5.7 and render it highly probable that the poor results with straw extracts mentioned above were due to a combination of too low a pH in the beginning (when the acetic acid had not yet been converted) and an insufficient aeration (for the latter point vide also sub c).

Some cultures on pure xylose and glucose, resp. xylose with a little glucose, acetic acid and acetic acid plus glucose, resp. and on glucose with a little acetic acid were carried out in 1943 (table II).

TABLE II.

Laboratory cultures on sugars.

Date 1943	Medium	Inoculation	Duration h	Yeast production g/l	Sugar content % before	Sugar content % after	Acetic acid %	Yield % of total substrate converted	Remarks
4/29 *a*	1.26 l with 3.05 g glucose and salts for 1.5 l. 255 ml with 26.85 g glucose added afterwards.	10.5 g from straw culture	9½	9.13	2.00[1])	0.00	—	46	aeration 20-80 l/h. Initial pH 5.9, after 1½ h decreased to 4.4 and kept between about 3 and 5.
4/29 *b*	1.5 l with 30 g xylose and salts.	as 4/29 *a*	13½	7.88	2.00	0.05	—	40	aeration 20-80 l/h. Initial pH 6.0, after 4 h decreased to 4.1 and kept between 3.5 and 5.5.
4/30 *a*	1.5 l with 30 g xylose, 3 g glucose and salts.	as 4/29 *a*	13½	8.51	2.20	0.03	—	39	aeration 20-80 l/h. Initial pH 6.2, for 3 h kept between about 5 and 6, then in 1½ h decreased to 4.2 and kept between 3.5 and 5.5.
4/30 *b*	1.5 l with 30 g xylose, 3 g glucose, 3 g acetic acid and salts.	as 4/29 *a*	13	9.47	2.20	0.02	0.20	40	aeration 20-80 l/h. Initial pH 5.8, for 4 h kept between 5 and 6, then in 3/4 h decreased to 4.9 and kept between 3.5 and 5.5.
5/18 *a*	1.26 l with 3.05 g glucose, 3.0 g acetic acid and salts for 1.5 l. 255 ml with 26.85 g glucose added afterwards.	10.5 g from 4/30 *b*	10½	9.98	2.00[1])	0.00	0.20	45	aeration 20-80 l/h. Initial pH 6.1, for 4¼ h kept between 5.5 and 6.5, then in 1 h decreased to 3.8 and kept between 3.5 and 5.5.
5/18 *b*	1.5 l with 30 g xylose, 3 g acetic acid and salts.	as 5/18 *a*	13½	8.52	1.92	0.01	0.20	40	aeration 20-80 l/h. Initial pH 6.0, for 4½ h kept between 5.5 and 6.0, then in 1¾ h decreased to 4.0 and kept between 3.5 and 4.5.

1) These figures represent the total amount of glucose added, calculated as % of the final amount of medium (i.e. 1515 ml).

4/29*a* is representative of a well-aerated glucose culture[1]). The yield is within the range given by LECHNER and ILLIG (16), the mean rate of conversion amounts to 2.11 g/lh. The parallel culture on xylose (4/29*b*) shows a lower yield and a much slower rate, *viz.*, 1.44 g/lh. Moreover, the conversion of the sugar is not complete. LECHNER (12) reports for xylose as a substrate the same yield as for glucose, but also mentions the lower rate and the incomplete conversion. During the conversion of the pure sugars the pH tended to decrease, chiefly owing to the consumption of NH_4-ions, and had to be adjusted frequently by the addition of ammonia, this tendency of course being stronger in the glucose than in the xylose culture.

The next set of parallel cultures (4/30*a* and *b* in table II) were made with xylose to which some glucose, and glucose plus acetic acid had been added respectively. In both cultures the yield[2]) is practically the same as that of the xylose culture 4/29*b*, but the mean rates of conversion (calculated on total substrate) are higher *viz.*, 1.61 and 1.83 g/lh resp. In both cultures the pH was first kept at a high level (5-6) and later on lowered by the natural acid production to a lower level (3.5-5.5), where it was kept by the addition of ammonia. However, in the xylose/glucose culture the initial high pH level had to be maintained by the addition of ammonia and in the xylose/glucose/acetic acid culture by the addition of hydrochloric acid which shows that in the latter culture the acetic acid is converted during the first hours of the culture (after exactly 4 h the tendency of the pH to rise changed into a tendency to fall). Moreover, attention is drawn to the fact that in the xylose/glucose culture the falling tendency of the pH from 3 to 4 h after the beginning was much smaller than either before or after this period. This indicates that in the beginning chiefly glucose was converted, whereas the conversion of the xylose did not reach its full rate until after 4 h.

[1]) In order to suppress alcohol production a glucose culture must be started with a dilute solution (0.5%), the bulk of the glucose being added in small portions in the course of the experiment. As xylose is not fermented to ethanol, the whole amount (2.0%) can be present from the beginning, without impairing the yield.

[2]) As it was found in cultures on straw extracts that the acetic acid was always converted completely, no determinations of residual acetic acid were carried out.

In the last set of parallel cultures (5/18*a* and *b*) glucose/acetic acid and xylose/acetic acid were compared as substrates. The yields of these cultures checked well with those of the cultures on the corresponding pure sugars. The rates amounted to 2.10 and 1.56 g/lh resp. i.e. about the same as found for pure glucose and xylose/glucose resp. Again the pH was initially kept at a high level and left to decrease to a lower one afterwards. In both cases the pH during the initial period showed a tendency to rise; the transition between a rising and a falling tendency occurred after 5 h in the xylose and after $4^1/_2$ h in the glucose culture. It should be pointed out, however, that in the xylose culture the rising tendency manifested itself practically from the beginning, whereas in the glucose culture it was preceded by a period of a weak, but distinct falling tendency which began right at the start of the culture and lasted for two hours. Such a falling tendency was not observed in the xylose/glucose/acetic culture 4/30*b*, but more than once in straw extract cultures (vide sub c). Apparently development in the glucose/acetic acid culture began with the conversion of the small initial amount of glucose, soon followed by that of the acetic acid. The remainder of the glucose was not added before the trend of the pH indicated that the acetic acid had been practically consumed.

The primary importance of these cultures with pure sugars lies in the establishment of the stimulating effect of small quantities of glucose or acetic acid, and especially of a combination of the two, on the conversion of xylose.

c. Experiments with straw extracts.

As there is no point in dwelling upon the erratic and mainly poor results obtained before May 1941, only those of later periods, during which the cultures were supplied with an adequate amount of air, will be discussed in more detail. The data pertaining to some representative cultures are collected in table III. It appears that an aeration of 10-40 l/h is sufficient for a good development and conversion in 2 l of extract in the apparatus employed and that yields of 45-50% of dry yeast, calculated on total substrate consumed (sugar plus acetic acid), may be obtained.

The first three cultures in table III show that the conditions of preparation of the extract do not influence the result. Cultures 117 and 118 demonstrate the harmful influence of an insufficient

TABLE III.

Laboratory cultures with straw hydrolysates.

Date 1941	Nr	Medium	Inoculation	Duration	Yeast production	Sugar content %		Acetic acid	Yield % of total substrate converted	Remarks
				h	g/l	before	after	%		
5/8	105	2.0 l wheat straw extract (1 h at 3 atm.), all salts.	14 g from straw culture	11½	9.00	1.86	0.17	0.22	47	aeration 8½-100 l/h. pH 1st. 5 h 5.5-6.0, afterwards 4.0-4.6.
5/9	107	2.0 l wheat straw extract (½ h at 3 atm.), all salts.	14 g from straw culture	10½	9.04	1.90	0.18	0.22	47	aeration 15½-55 l/h. pH 1st. 5 h 5.5-6.0, afterwards 3.9-4.7.
5/12	109	2.0 l wheat straw extract (¾ h at 2 atm.), all salts.	14 g from 107	10	9.28	1.90	0.24	0.21	50	aeration 12½-37½ l/h. pH 1st. 5 h 5.5-6.0, after 1 h decreased to 4.5 and kept between 4.0 and 4.6.
5/27	116	2.0 l wheat straw extract (1 h at 3 atm.), no K_2SO_4 and $MgSO_4$.	14 g from 114	10	9.52	1.95	0.28	0.22	50	aeration 10½-47½ l/h. pH 1st. 6 h 5.5-6.4, after 1¼ h decreased to 4.3 and kept between 3.8 and 5.0.

TABLE III (continued).

5/28	117	2.0 l wheat straw extract (1 h at 3 atm.), all salts.	14 g from 114	14½	7.88	1.95	0.25	0.22	41	aeration 1st. 9 h about 1/3 of that of 118, afterwards 60-90 l/h. pH 1st. 6¼ h 5.5-6.0, after 1¾ h decreased to 4.4 and kept between 3.9 and 4.6.
5/28	118	as 117.	14 g from 114	9	8.56	1.86	0.25	0.23	47	aeration about as 116. pH 1st. 5¾ h 5.3-6.2, after 1 h decreased to 4.4 and kept between 4.2 and 5.1.
6/3	124	2.0 l rye straw extract (1 h at 2 atm.), no K_2SO_4 and $MgSO_4$.	28 g from 120	8½	7.08	1.74	0.27	0.17	43	aeration 38½-110½ l/h. pH 1st. 4½ h 5.5-6.2, after 1 h decreased to 4.1 and kept between 4.0 and 4.9.
6/4	125 I	2.0 l wheat straw extract (1 h at 3 atm.), no K_2SO_4 and $MgSO_4$. 1.3 l drawn off at end.	14 g from 122	7½	8.36	2.02	0.34	0.27	43	aeration 31-231-114 l/h. pH 1st. 4¾ h 5.5-6.1, after 1 h decreased to 4.2 and kept between 4.0 and 4.8.
6/4	125 II	Extract as 125 I. 1.6 l fresh extract added to remainder of 125 I.	± 500 ml of 125 I	6	5.68	1.58	0.44	0.21	43	aeration 83-168-152 l/h. pH 1st. 3 h 5.5-6.2, after 1½ h decreased to 4.2 and kept between 4.1 and 4.5.
4/2 1943	—	2.0 l wheat straw extract (1 h at 3 atm.), no K_2SO_4 and $MgSO_4$.	14 g from glucose 3/30'43	10¾	9.11	2.21	0.35	0.27	43	aeration 20-80 l/h. pH 1st. 5¼ h 5.3-6.5, in 1 h decreased to 4.4 and kept between 3.8 and 4.6 and for the last h between 5.5 and 6.0.

aeration: the yield, and especially the rate of conversion (1.32 and 2.05 g/lh for 117 and 118 resp.) are lower for the less well-aerated culture. The rates of most of the other cultures vary from 1.66 to 2.05 g/lh, being only higher in the excessively aerated cultures 125 I and II (2.60 and 2.25 g/lh resp.). Leaving the two latter cultures and 117 out of consideration the mean of the rate values of the other 7 cultures amounts to 1.89 g/lh, i.e. nearly the same as that of the xylose/glucose/acetic acid culture mentioned sub b (1.83 g/lh). Cultures 116 and 124 ff. show that potassium and magnesium salts can be omitted from the medium; apparently these are provided in sufficient amounts by the extracts themselves. It appears from culture 124 that rye straw as a raw material yields practically the same results as wheat straw.

Cultures 125 I and II demonstrate that it is quite feasible to use a portion of a finished culture as the inoculum for the following one, without separating the yeast from the medium.

The last culture in table III was made with the extract of 1-27-43 mentioned in chapter 2.

A peculiar feature of the straw extract cultures is that a certain amount of the reducing sugar (about 0.2-0.4%) is not converted by the yeast, notwithstanding the fact that, judging from the trend of the pH at the end of the culture (which remained constant for $^1/_4$-$^1/_2$ hour), the conversion had come to a standstill.

As already stated on p. 106, the pH in these straw extract cultures was kept between approximately 5.5 and 6.0 for the first few hours (i.e. until the trend of the pH showed that the acetic acid had been consumed) and was then lowered by the natural acid production of the culture to 4.0-4.5, where it was kept for the remainder of the duration[1]). As in the cultures on xylose with acetic acid mentioned sub b, the change of a rising tendency of the pH into a falling one was taken as a criterion that the acid had practically disappeared. The trends of the pH in the cultures listed in table III are given below (↑ denotes a rising, ↓ a falling tendency, the figures denote hours after the start of the culture):

[1]) When the pH was kept at 5.5-6.0 throughout the culture, considerable development of bacteria occurred.

105		↑ 0 — 5	↓ $5\frac{1}{2}$—$10\frac{1}{2}$
107	↓0—$1\frac{1}{4}$	↑ $1\frac{1}{4}$— 4	↓ 4 —10
109	↓1—2	↑ 2 — $3\frac{1}{2}$	↓ $3\frac{1}{2}$— $9\frac{1}{2}$
116	↓0—$2\frac{1}{2}$	↑ $2\frac{1}{2}$— 5	↓ 5 — $9\frac{1}{2}$
117	↓0—$8\frac{1}{2}$	↑ $8\frac{1}{2}$—$10\frac{1}{4}$	↓$10\frac{1}{4}$—14
118	↓0—$2\frac{1}{2}$	↑ $2\frac{1}{2}$— $4\frac{3}{4}$	↓ $4\frac{3}{4}$— $8\frac{3}{4}$
124	↓0—$1\frac{1}{2}$	↑ $1\frac{1}{2}$— $2\frac{1}{2}$	↓ $2\frac{1}{2}$— 8
125I		↑ 2 — $4\frac{1}{4}$	↓ $4\frac{1}{4}$— $7\frac{1}{2}$
125II		↑ 0 — $2\frac{1}{2}$	↓ $2\frac{3}{4}$— 6
4/2'43	↓0—$1\frac{3}{4}$	↑ $2\frac{1}{4}$— $4\frac{1}{2}$	↓ $4\frac{1}{2}$— $9\frac{1}{2}$
			↓ $9\frac{3}{4}$—$10\frac{1}{4}$

These figures show that considerable diversity exists. Nevertheless, in the majority of cases the pH first showed a tendency to fall during the first 1-2 hours (in the underaerated culture 117 this period lasted much longer), followed by a tendency to rise for about the same time, and a pronounced tendency to fall during the last 3-6 hours of the culture. As the conversion of acetic acid will exert a far greater influence on the pH than the conversion of sugar (one base equivalent being formed for every molecule of acid consumed), it may be safely assumed that no appreciable amounts of acetic acid are converted outside the period of a rising tendency of the pH. However, it is quite possible that during this period some sugar is converted along with the acetic acid. In culture 4/2'43 the pH, after having remained constant from $9\frac{1}{2}$-$9\frac{3}{4}$ h after the beginning, was brought back from 4.3 to 5.5-6.0, after which it again showed a falling tendency for about $\frac{1}{4}$ h and remained constant during an additional $\frac{1}{4}$ h. Notwithstanding this, an appreciable amount of reducing sugar remained unconverted in this culture.

Bearing in mind the composition of the straw extracts and the experience obtained with cultures on pure sugars discussed sub b, it seems justified to draw the following picture of the course of the conversion of the assimilable constituents of the straw extracts. The yeast begins to attack the small amount of glucose present in the extracts (falling tendency of the pH) and when development has set in it also attacks the acetic acid; conversion of the xylose does not set in before an appreciable part of the acetic acid has been consumed. This means that glucose and acetic acid act as „pace-makers" for the conversion of xylose. Under certain (as

yet unknown) conditions conversion of the acetic acid may set in right at the start of the culture; in a few instances (not listed in table III) little or no change in pH occurred during the first few hours, indicating that acid production by conversion of sugar was balanced by the production of alkali by conversion of acetic acid.

d. Additional experiments on the course of the conversion in straw extracts and on the influence of the pH.

Though it is hoped to publish a separate, detailed account of these experiments, their results are so closely connected with the ones mentioned sub b and c that it is deemed advisable to give a short exposé of these results in the present paper.

In the first place the course of the conversion of sugar and acetic acid in a straw extract culture was determined by analysis of samples drawn at 2 h intervals. The following figures were found:

Time in h	Sugar %	Acetic acid %
0	1.90	0.19
2	1.72	0.17
4	1.54	0.09
6	1.28	0.01
8	1.06	0.00
10	0.75	0.00
12	0.32	0.00
14	0.19	0.00

It appears that during the first 2 h an appreciable amount of sugar was converted but only little acetic acid. During the next 4 h the bulk of the acetic acid was converted, with a concomitant sugar conversion at about the same rate as during the first 2 h. Shortly after completion of the conversion of the acetic acid the rate of sugar conversion increased. This acceleration of the sugar conversion after disappearance of the acetic acid (indicated by the change of a rising tendency of the pH into a falling one) was also observed in a number of earlier straw extract cultures in which the sugar content was determined every 2 h. In some cases a slight decrease in the rate of sugar conversion during the „acetic acid" period (i.e. that of a rising tendency of the pH) was observed.

A quite divergent trend of the pH was observed in a culture on an extract containing 1.01% of sugar and 0.11% of acetic acid, and lasting 6½ h. Neither at a pH of 5-6, nor at 4-5 did the pH show any but a very slight tendency to change in either direction. The yeast yield in this aberrant culture was quite normal, *viz.* 45%.

The optimum pH for conversion of acetic acid in straw extract appeared to be 6.0, for the sugar no clear-cut results were obtained. It was also found that, with a moderate aeration (50-60 l/h for a 2 l culture), no conversion of acetic acid took place at a pH of 4.2-5.0, whereas, with an air supply of 110 l/h the rate of conversion of the acetic acid was much higher and little difference was found between the rates at pH 4.3 and 5.5.

In a certain straw extract culture the sugar conversion stopped after 10 h (as judged from the constancy of the pH at 4.75) at a sugar content of 0.50%. After raising the pH to 6.6 the conversion started anew and stopped again after 3½ h; the sugar content amounted then to 0.18%.

Mention should also be made of an experiment with an extract obtained by total saccharification of straw with superconcentrated hydrochloric acid. The extract contained 2.00% of sugar and practically no acetic acid (this having been distilled off together with the hydrochloric acid after the extraction). Notwithstanding the fact that the sugar in this extract consisted for about 3/5 of glucose and for about 2/5 of xylose and that the culture was started with the total amount of the undiluted extract, no indications of alcoholic fermentation were observed. The initial pH was 5.0 and was kept between 4.6 and 4.8 by the addition of ammonia. Already after 3½ h the pH did not decrease any more, although the medium still contained 0.98% of sugar. After bringing the pH to about 5.8 conversion started again and the concentration decreased in another 3 h to 0.36%. The yield amounted to 39.3%.

4. Experiments on a semi-technical scale.

a. Concise description of the apparatus employed (fig. 2).

The complete apparatus consisted of:

1. The rotary digester described in chapter 2.
2. a cylindrical tank (200 l) with a coarsely perforated plate inserted above the conical bottom, and covered with a more finely perforated plate (diameter holes 2 mm). On the latter

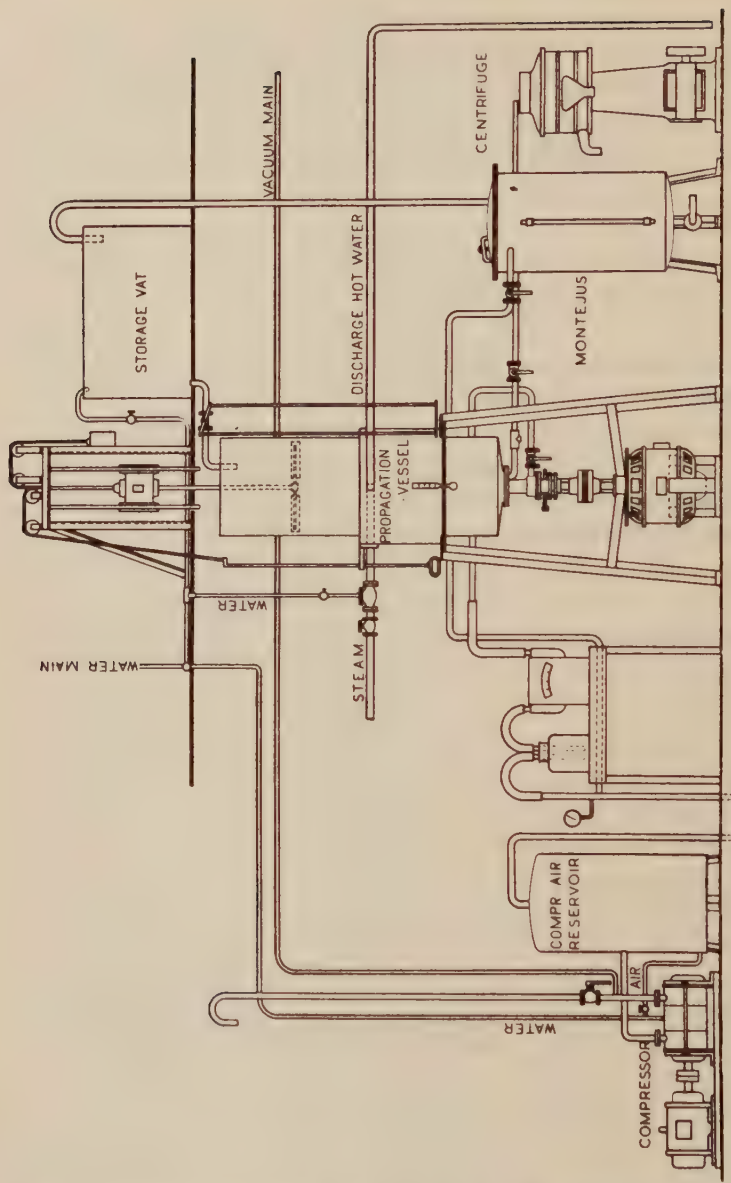
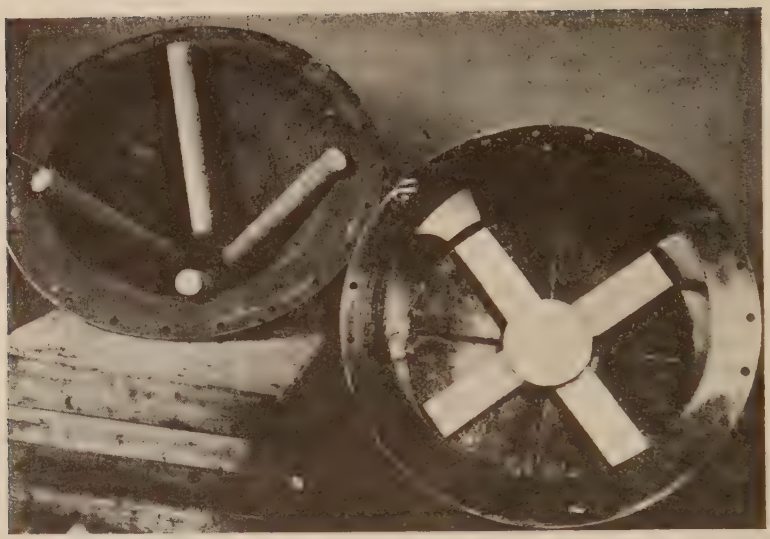


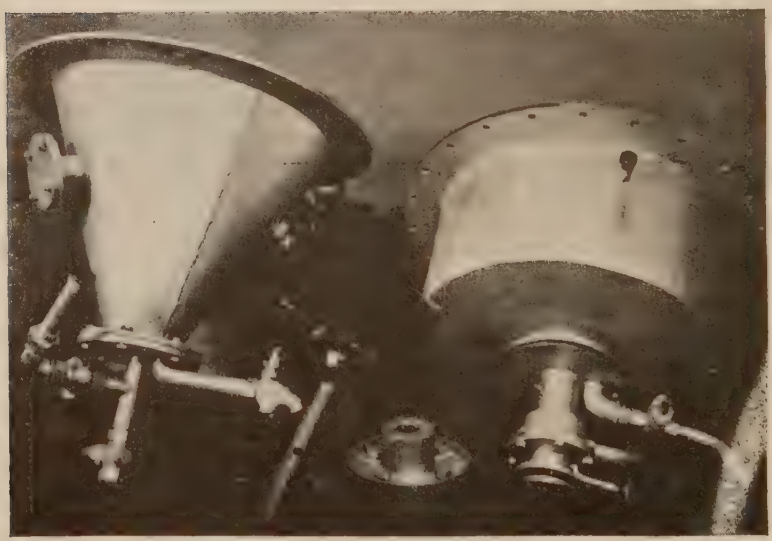
Fig. 2. Apparatus for growing food yeast on a semi-technical scale. The lower part of the yeast propagation vessel contains the „Vogelbusch" apparatus, which is rotated by means of the electric motor. The fan inside the tank is used to disperse foam and is driven by a small electric motor. This device can be raised or lowered with a handle, to accomodate it to the height of the wort and the foam level.

a sheet of filter paper was spread, which was protected by placing a piece of wire screen on top of it. The contents of the digester were transferred to this tank through the man-hole in the top. The extract was then squeezed out by compressed air and the residue washed twice with 50 l of water. The filtrate was transferred to

3. a montejus, in which the straw extract was collected, mixed with nutrients, heated or cooled to 30°C. and forwarded with compressed air to
4. a storage vat, with a capacity of 400 l, installed in the attic of the semi-technical laboratory right above the propagation vessel.
 From this storage vat, which was covered with an iron lid, the wort flew into the
5. propagation vessel with a capacity of 400 l which consisted of a cylindrical upper part of 60 × 135 cm, connected by a flange and bolts to a conical bottom part of 60 cm diam. and 50 cm height, containing four aeration elements of porous stoneware. This bottom part could be replaced by a cylindrical one containing the „VOGELBUSCH" aeration device (see below). About one third of the propagation vessel was provided with a double wall connected to the water supply, to a steam injector and to a water outlet. By this device the medium in the vessel could be heated or cooled and kept at 30°C. during the experiments.
6. The VOGELBUSCH aeration device (30). This consisted of a vertical hollow axis, guided through the bottom of the vessel and bearing at its upper end a propeller with four horizontal streamlined hollow blades with small holes in their lower sides. The apparatus was directly coupled to an electromotor of 7.5 H.P., while through its hollow axis air was introduced into the wort. As soon as the air bubbles came out of the propeller holes they were snatched off by the friction with the surrounding liquid. In order to prevent the liquid from being carried along by the propeller a number of iron strips was fixed to the tank wall, beneath as well as above the propeller.

In this way the wort was aerated with numerous extremely fine air bubbles; the energy used up by the friction between the propeller blades and the liquid at 300-400 revolutions per minute amounted to ± 2 H.P. per hour. Though the capacity of the aeration apparatus amounted to 3600 l.p.h., the maximum quantity which could safely be handled without too much trouble caused by foaming was not more than about 1500 l.p.h. As an alternative another device could be used, which consisted of the conical bottom part with four aeration candles of porous stoneware (diam. 5 cm, length 45 cm). Both aeration devices are shown in the appended photographs (Figs. 3 and 4). Especially with glucose as a substrate





Figs. 3 and 4. The aeration apparatus.
Left: conical bottom piece of the propagation vessel with four large porous candles. Right: „Vogelbusch" aeration device. Note the perforated, four-bladed fan in the middle of Fig. 4 and the four iron strips to prevent whirling of the wort in Fig. 3.

the results obtained with this apparatus lagged behind those achieved with the VOGELBUSCH apparatus, which proved to be very effective and gave higher yields with less air. Moreover the friction between propeller and wort created a vacuum inside the propeller, which was sufficient to introduce the air so that the compressor could be put out of action.

Mechanical foam control.

During the experiments on a semi-technical scale the use of chemicals for foam control proved to be too expensive and had to be limited to emergency cases. From the different mechanical methods used to this end, the following may be mentioned.

A rotating Archimedean screw, which forced the wort above the foam level and sprayed it on the foam; the effect was inadequate and the device could only effectively be used with a glucose solution, which foamed less than straw extract.

Also spraying the foam with wort with the aid of a spray nozzle and compressed air proved unsuccessful.

An adequate solution of the foam problem consisted of a fan, made of two nearly horizontal blades of strong wire gauze (meshes 2 × 2 mm) 20 × 20 cm rotating at a speed of 1400 rev. p. minute on a vertical axis.

Motor and fan could be raised or lowered by means of a handle (see fig. 2) and had to be adjusted just above the upper level of the foam. In that case the foam was flung with great force against the wall of the propagation vessel which caused the air bubbles to explode, whereupon the wort flew back into the vessel[1]).

Care had to be taken that the foam did not rise above the fan, in which case the increased friction reduced the rotating speed to circa 60 r.p.m. which was insufficient to control foaming and endangered the motor. With normal straw extracts the device worked quite satisfactory and solved the foam problem in a rather simple way.

Yeast separation.

To separate the yeast from the mash a small de Laval centrifuge was used, which, however, was not a special yeast centrifuge and deposited the yeast in a solid layer against the side wall of the bowl. When it was desired to separate all the yeast from one charge, it had to be emptied twice or thrice.

pH Control.

Though originally an electrical pH control along similar lines as in the laboratory experiments was planned, it was soon proved

[1]) The principle of the apparatus was suggested by Ir. F. MULLER, mechanical engineer.

that this would be impossible, due to the stray currents originating from the electric motors connected to the propagation vessel. Therefore, the pH had to be determined with the aid of a quinhydrone electrode in small samples taken at regular times from the wort. When the wort became too acid or too alcaline ammonia, resp. hydrochloric acid, was added through a long tube, which ended in the lower part of the tank below the foam zone.

Accessories.

Compressed air (0.25 atm.) was delivered by a Sihi waterring compressor which was connected to a storage tank of 200 l. The quantity of compressed air used was measured by means of a large gas meter.

b. Single experiments.

The data pertaining to semi-technical cultures are listed in table IV. The first three were glucose cultures, that of 4/16 aerated by the porous candles, the other two by the VOGELBUSCH device. Glucose being a rather scarce product at that time, the first two cultures were made with about half the percentage used in the laboratory experiments. As in these experiments, the cultures were started with a very dilute solution, the remainder of the glucose being added later on in the form of small portions of a relatively concentrated solution. In all three experiments satisfactory yields were obtained. In the culture with porous candles 20-50 l of air per min. were used, in two other cultures with these candles (not mentioned in table IV) 10-25 l/min. were given which resulted in markedly lower yields, *viz.*, 37 and 36%. With the VOGELBUSCH device at 400 r. p.m., however, the latter amounts of air appeared to be sufficient for a satisfactory yield which shows this device to be superior to the 4 porous candles, both as used in our experiments.

The next two cultures in table IV are examples of single experiments with straw extract. Foaming could be adequately controlled by the rotating screens. The amount of air could be decreased to 6-17 l/min. and the number of r.p.m. to 300, without impairing the yield, presumably because the air bubbles in the straw extract were much finer than in the mineral glucose solution. A difficulty peculiar to the semi-technical experiments was caused by the stability of the foam when aeration was stopped at the end of the experiment. As the yeast tends to accumulate in the foam, it was difficult to draw a reliable sample for the determination of the

TABLE IV.

Semi-technical cultures with glucose and straw extracts.

Date 1941	Apparatus and medium	Inoculation	Duration	Yeast production	Sugar content %		Acetic acid	Yield % of total substrate converted	Remarks
					before	after	%		
4/16	Porous candles. 130 l 0.25 % glucose and salts for 176 l, 46 l 3.17 % glucose solution added afterwards.	1.5 kg from glucose 4/10-41	6¼	4.88	1.03[1])	0.00	—	47	aeration 20-50 l/min. Initial pH 5.4, after 1 h decreased to 4.3 and kept between 3.5 and 5.5.
5/6	VOGELBUSCH. 147 l 0.22 % glucose and salts for 180 l, 33.5 l 5 % glucose solution added afterwards.	1.5 kg from glucose 5/5-41	6½	5.44	1.11[1])	0.00	—	49	aeration 10-25 l/min. Initial pH 5.8, after 1¼ h decreased to 4.0 and kept between 3.7 and 5.
5/14	VOGELBUSCH. 130 l 0.14 % sugar and salts for 200 l, 73 l 5 % glucose solution added afterwards.	1.5 kg from glucose 13/5-41	9¼	8.68	1.84[1])	0.05	—	47	aeration 10-33 l/min. Initial pH 6.8, adjusted; after 1¾ h decreased to 4.9 and kept between 3.4 and 5.5.
6/10	VOGELBUSCH. 200 l wheat straw extract (1 h at 3 atm.), no K_2SO_4 and $MgSO_4$. Single culture.	1.5 kg from straw 6/9-41	8½	7.12	1.81	0.42	0.21	45	aeration 11½-32 l/min. pH 1st. 5 h 5.0-6.5, after 1 h decreased to 4.3 and kept between 4.1 and 5.
6/11	VOGELBUSCH. 180 l, for the rest as 6/10.	1.5 kg from 6/10	8¼	7.16	1.76	0.44	0.22	47	aeration 5.8-18.8 l/min. pH 1st. 6 h 5.0-6.3, after ¾ h decreased to 4.6 and kept between 4.2 and 5.
6/16-17	VOGELBUSCH. 135 l as 6/10. Serial culture.	± 45 l from culture 6/11	9¼	10.48	2.31	0.34	0.30	46	aeration 11.6-21.6-19.5 l/min. pH 1 st. 5¾ h 5.6-6.3, after 1 h decreased to 4.4, kept for 1½ h at 3-5 and further between 5 and 6.
6/18	as 6/16-17.	± 78 l from culture 6/16	11¼	8.88	2.41	0.52	0.32	40	aeration 7.8-24.9 l/min. pH 1st. 6½ h 5.5-6.4, after 1½ h decreased to 4.3, kept for 1½ h at 4.3-5.5 and further between 5.3 and 6.5.

1) See footnote of table II.

final yeast content. However, at least in the single experiments, this difficulty could be overcome by decreasing the speed of the VOGELBUSCH wheel to 250 r.p.m. (at which speed it did not deliver any air into the medium, but neither allowed the medium to enter into the aeration system) and slowly lowering the rotating wire screens, thus gradually destroying the foam layer on top of the medium.

The yields and the rates of conversion of these cultures are quite similar to those of the laboratory cultures in table III. Attention is drawn, however, to the high final sugar contents, a phenomenon also noticed in the laboratory cultures 125 I and II. The trends of the pH were similar to those observed in the laboratory cultures, but the relative amounts of 20% HCl and 10% NH_4OH which had to be added were smaller in the semi-technical cultures. This shows that relatively more sugar was converted simultaneously with acetic acid than in the laboratory cultures. As a mean of the four last single experiments a yield of 660 g of dry yeast was obtained from 10 kg air-dry straw (moisture content abt. 15%).

c. Serial experiments.

The investigation was concluded with 10 serial experiments, two of which are listed in table IV. The first eight ones were run with the VOGELBUSCH device and the two last ones with the porous candles. The first experiment was started on 6/16 '41 at 9.30 and the last one with the former aeration device concluded at 6/19 at 18.45, so that the mean duration amounted to 10 h. Immediately after the last experiment the VOGELBUSCH device was changed for the candles. The two experiments with the latter lasted $7\frac{3}{4}$ and $6\frac{1}{4}$ h resp. The work was carried out in three shifts of two men each, which man power proved to be amply sufficient (however, one man per shift would have been inadequate).

The extracts were prepared as described in chapter 2. The extracts were neutralized by adding 2.0 l of 15% milk of lime to the digester after blowing off and by rotating for another 10 min. The total content of the digester (two batches) was brought into the pressing tank and the extract separated from the residue with compressed air of 1.7 kg/cm², the extract being forwarded directly into the montejus. The residue was washed twice with 40-60 l of cold water, the washings being also pressed into the montejus. The nutrient salts were dissolved into 5-10 l of water

and added to the montejus, where the medium was brought to the desired volume; it was then forwarded to the storage vat above the propagation vessel. The first serial culture was inoculated in the storage vat with 1.5 kg of wet yeast from the last single experiment, while the other cultures were inoculated with a certain volume of the preceding ones. As it took too long a time for the foam to subside at the end of a culture so that the volume of medium to be drawn off could be measured, this amount was measured by weighing in buckets which were emptied into the drainage vessel from which it was pumped into the centrifuge after further subsidence of the foam. The centrifuged yeast was spread on large sheets of strawboard and dried on top of the boilers of the factory. Throughout these experiments the yeast had a healthy appearance and only a small number of bacteria were noticed so that this procedure can probably be adopted for a technical manufacture without danger of contamination of the yeast.

The two serial experiments listed in table IV are quite similar to the single experiments, with the exception of the somewhat lower yield of the last one. However in the other experiments (both those with the VOGELBUSCH device and those with the porous candles)still lower yields (27-36 and 32 and 11 % resp.) were obtained. The chief cause of these lower yields must probably be sought in the difficulties in drawing reliable samples for the yeast determinations as outlined sub b. Also some loss of yeast may have occurred in foam which was blown out of the tank between the rim and the cover by the air currents set up by the rotating wire screen. Since the mean rates of conversion of sugar plus acetic acid were essentially the same as those found in the laboratory cultures, it is considered improbable that these low yields were caused by some inhibition of the development of the yeast.

d. Properties of the yeast produced.

The primitive method of drying mentioned sub c. was the only one available for conservation of the yeast for analytical purposes. It proved to be impossible to determine the ash content by direct combustion so that the sulfate ash content was determined instead. The protein content was determined by multiplying the nitrogen content with 6.25[1]). The sulfate ash content varied from

[1]) The methods employed are described in (21), p. 57 and (22), p. 33 resp.

3.3 to 22.1%, that of protein from 18.6 to 52.2% The variation in the ash content is probably due to the fact that the yeast had not been washed (which would have taken up too much time with the available apparatus). The variation in the protein content is less easy to explain, but also here the results may have been influenced by the primitive method of handling the yeast. At any rate the highest values obtained (41.0, 49.5 and 52.2) are well in line with those given by LECHNER (12) for *Torulopsis utilis* grown on xylose.

Summary.

1. Cereal straw was partially hydrolyzed with dilute sulphuric or hydrochloric acid at elevated temperatures, yielding about 20% of matter assimilable by *Torulopsis utilis* (calculated on oven-dry straw), which consisted chiefly of xylose, with small amounts of glucose and acetic acid.
2. Experiments on a laboratory scale with non-fermentable substrates like ethanol and acetic acid showed that also in these cases an ample aeration is an essential condition for a good yield.
3. Acetic acid is harmful to the yeast, even in small concentrations, when the pH is lower than 5.0.
4. Small quantities of glucose or acetic acid stimulate the conversion of xylose by *Torulopsis utilis*.
5. With an adequate aeration and an initial pH of 5.5-6.0 (maintained at this level until the acetic acid has disappeared and then lowered to 4.0-4.5) satisfactory conversion rates and yields could be obtained on straw extracts as mentioned sub 1.
6. The results of the laboratory experiments mentioned sub 5 could be reproduced in a semi-technical installation with a capacity of 200 l straw extract.
7. Good results were obtained in this installation with a VOGELBUSCH aeration device, while foaming could be adequately controlled by means of a rotating wire screen.

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INFECTION WITH *SALMONELLA ANATUM* OF THE COW IN CONNECTION WITH SALMONELLOSIS OF DUCKS

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(Received April 30, 1948).

1. Introduction.

With regard to the literature about the occurrence of *S. anatum* we refer to the monograph of KAUFFMANN (2) and the text-book of TOPLEY and WILSON (3). According to these *S. anatum* has been found for the first time in ducks, afterwards in man, fowl, turkey, pig, silverfox and in the cat. BRUNER and EDWARDS (1) could demonstrate this *Salmonella* species in dogs. In Great Britain during the last war several outbreaks of food poisoning were found to be due to *S. anatum*. Evidence was brought to show that *Salmonella* infection of dried egg was responsible for these outbreaks. From 840 strains of *Salmonella*, isolated from dried egg, 30 belonged to the anatum type (4).

So far as we are aware no *S. anatum* has previously been described from cattle.

In March, 1948, we received for bacteriological examination pieces of meat, spleen, kidney and liver, and also the gall-bladder of a cow, which had been slaughtered in emergency. At the post-mortem examination some gas was found in the subcutaneous connective tissue of the left side of the thorax and in the muscles of the left shoulder. In the stomach there was a large quantity of red-brown, fetid liquid, while the intestine was almost empty. In the uterus was found an emphysematous foetus.

From the liver and the gall-bladder *S. anatum* was isolated. The meat, the spleen and the kidney proved sterile.

At the same farm there were also some 300 ducks, which lived at a short distance (10—20 M) from the cow-shed. In 13 of the 70 samples of faeces gathered from the ducks the same *Salmonella* strain was isolated as from the liver and the gall-bladder of the

cow. In the faeces of the remaining 8 cows no *Salmonella* bacteria could be demonstrated.

The cattle and the ducks were taken care of by the same person. It was obvious therefore, that the infection of the cow in question was caused by the contaminated faeces of the ducks.

2. Examination of the *Salmonella* strains.

The organisms, cultivated from the liver and the gall-bladder of the cow and the faeces of the ducks, were motile rods, which possessed the usual cultural and biochemical properties of the *Salmonella* group. Glucose, maltose, arabinose, rhamnose, trehalose, xylose, galactose, fructose, mannitol and dulcitol were fermented within 24 hours. Lactose, saccharose, raffinose, inositol and adonitol were not attacked. H_2S was produced but indol was not formed nor was gelatin liquefied.

On serological examination, which was performed according to the directions of KAUFFMANN (2) the organism showed a positive slide agglutination-reaction with O-sera of *S. london* (III. X.XXVI), *S. newington* (III . XV), *S. senftenberg* (I . III. XIX) and *S. minnesota* (XXI . XXVI). The tube agglutination-reaction was positive with O-serum of *S. london*, which had been absorbed with *S. minnesota* and *S. newington*, in a dilution of up to 1 : 160. Consequently the O-antigens are III . X . XXVI.

The H antigens of the culture were diphasic. In the slide agglutination test phase 1 gave a positive reaction with *S. eastbourne*, phase 1 (e, h) serum. Phase 2 was agglutinated by serums for different non-specific phases of the genus (1,2—1,2,3—1,5 — 1,6 and 1,7). When tested with single factor serums for antigens 2, 3, 5, 6 and 7, it was agglutinated only by 6 serum. Consequently the antigenic formula of the examined strains was: III . X . XXVI: e, h — 1,6 (*S. anatum*).

Summary.

S. anatum was cultivated from the liver and the gall-bladder of a cow, slaughtered in emergency. Among the ducks of the same farm an infection with the same *Salmonella* type was found. It is obvious that the infection of the cow was caused by the contaminated faeces of the ducks.

So far as we are aware no *S. anatum* has previously been described from cattle.

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(From the Laboratory of Parasitology of the University and the Department of Parasitology of the Institute of Tropical Medicine at Leiden).

THE ROLE OF FROGS IN THE EPIDEMIOLOGY OF WEIL'S DISEASE

by

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(Received June 15, 1948).

Little attention has been paid to the rôle that frogs might play in the epidemiology of Weil's disease. However even before the significance of the rat as carrier of leptospirae was known, HÜBENER and REITER (6), UHLENHUTH and FROMME (15) inoculated, without mentioning any further detail, frogs with virulent Weil-leptospirae, but they either were not able to establish any pathogenic activity or when the frogs had died no leptospirae could be detected in the organs. These authors thus found frogs to be „refraktär gegen die Krankheit". PETTIT (10) writes with respect to this „natural immunity:" *Rana esculenta* est capable d'altérer légèrement les Spirochètes". UHLENHUTH and FROMME detected sometimes in the intestine of frogs spirochaetes of the Weil-type.

As merely sparse data have been gathered a further extension of our knowledge of this matter is very desirable; this has been attempted at in the following.

I. THE PRESENCE OF ANTOBIDIES WITH RESPECT TO PATHOGENIC AND APATHOGENIC LEPTOSPIRAE IN THE SERUM.

The blood of frogs, caught in different ditches, in different parts of our country and on different moments, was obtained by withdrawing it under ether anaesthetic from the big skin- and foreleg venes and from the heart. The tests for agglutinins and lysins, which in the following will serve as a measure for the degree of immunity, were made as is customary in the diagnostic of Weil's disease, except that here also 1 : 2, 1 : 4, and 1 : 8 serum dilutions were examined. When the end titre was feebly positive it was

recorded as positive. The tests were made with the following pathogenic strains: the incomplete (strain Coppée or Kantorowicz) and complete (strain Wijnberg) biotype of *Leptospira icterohaemorrhagiae*, with *L. canicola* (strain Polly), *L. grippotyphosa* (strain Andaman) and with *L. sejrö* (strain Sejrö). The separate sera could not be agglutinated with all these strains, the research having been started already many years ago when all the above species of Leptospira and the existence of biotypes of a single species were not known as yet. Moreover we came into possession of one definite strain at a later date and the quantity of serum derived from one frog was often insufficient for agglutination with all of the strains. Besides these strains, 10 strains of the apathogenic saprophytic *L. biflexa* (*pseudoicterogenes*), isolated from tap-water or ditch-water, were tested. The result is presented in the tables I and II. The tests recorded in table I bear on 99 specimens of *Rana fusca* and 10 specimens of *Rana esculenta*. The numbers after the „+" indicate *R. esculenta*. Table II bears on 21 specimens of *Rana fusca*.

Table I.

	Number of times that serum was agglutinated with the strains:				
Titer	Coppée Kantorowicz	Wijnberg	Polly	Andaman	Sejrö
negative	0 + 2	10 + 1	1 + 1	11	12 + 7
1 : 4	4	18 + 1	2	6	1
1 : 8	1 + 2	5 + 2	2	0	0 + 1
1 : 10	14 + 2	11 + 3	4 + 2	2 + 1	1 + 1
1 : 25–50	38 + 2	2 + 2	11 + 2	0 + 2	—
1 : 100–150	36 + 2	—	6	0 + 2	—
1 : 250–300	6	—	—	—	—

Tabel II.

	Number of times that serum was agglutinated with strains of *L. biflexa*:									
Titer	I	II	III	IV	V	VI	VII	VIII	IX	X
negative	1	0	0	0	0	0	0	0	1	1
1 : 4	0	0	0	0	0	0	0	0	0	0
1 : 8	0	0	0	0	0	0	0	0	1	1
1 : 10	3	1	0	0	1	0	1	1	2	11
1 : 25–50	11	8	6	6	2	5	5	8	13	7
1 : 100–150	4	—	3	3	6	4	3	—	3	1
1 : 250–300	1	—	—	—	—	—	—	—	—	—

These results indicate that:

a. A high percentage of frog sera reacts positively against many pathogenic leptospirae strains.

b. This reaction is highest (100 per cent) against the incomplete biotype of *L. icterohaemorrhagiae.*

c. A great difference in reaction exists between both biotypes of *L. icterohaemorrhagiae.*

d. The reaction with *L. canicola* is like the one with the incomplete biotype of *L. icterohaemorrhagiae.*

e. *L. grippotyphosa* and *sejrö* show no reaction or a very slight one.

f. No reason exists for the assuming of a difference in properties between the sera of both examined frog species.

g. The agglutinability exists also against strains of *L. biflexa.* In this respect no difference existed between the 10 strains examined.

h. The titers of the separate sera for the different strains did not agree regularly, nor did those for the apathogenic strains when compared with the pathogenic ones.

i. The reaction offers the picture of a typical agglutination, such as it is known in the lower dilutions of Weil immune sera of man, rabbit, rat and guinea-pig. Never (see also under 2) „lysisballs", known from higher dilutions of Weil immune sera, were observed.

j. Like the picture of the agglutination in immune sera of mice as recorded by Appelman, when compared with the picture in immune sera of man, shows a somewhat different type of its own (rather compact bands tapering on both sides), so it is the case in normal frog sera. Here a more or less thready structure, such as from a cobweb, occurs.

When in a same serum different titers may be noted for different leptospiral strains, it has to be kept in mind, that one antigen may need much more antibodies for agglutinating than another, so that the presence of a higher titer does not prove that more agglutinins are actually present (Dunlop (4)).

In connection with the above described agglutinability for leptospiral strains, it was interesting to examine whether this phenomenon would occur also for bacteria. For that purpose the action of 5 frogs sera on typhoid and paratyphoid A and B bacteria was studied. Although these sera agglutinated the Kantorowicz strain of *L. icterohaemorrhagiae* up to a titer of 1 : 10, the reactions

with the above bacteria gave a negative result in the serum dilutions 1 : 2 till 1 : 50. The agglutinating faculty of frog sera is not the same therefore for all kinds of microbes.

II. The frog as „carrier" of leptospirae and its reaction on the injection of virulent leptospirae.

Just as the rat excretes pathogenic leptospirae with its urine and functions thus as a carrier of the virus, so the frog might be conceived to act in the same way. This is not the case however. This may be derived from the fact that by inoculating in mice kidneys of 10 normal frogs, the blood of which was strongly positive with regard to Weil leptospirae, no pathogenic leptospirae were detected, nor were they found microscopically by means of dark field illumination. Brains were also examined, as *L. schüffneri* has

Table III.

Number of frogs	Quantity of inoculated culture of *L. icterohaemorrhagiae*	Mode of injection	Number of days after injection on which examination was made.	Result				
				Titer of blood for strain Coppée	Injection in mice of:			
					blood	liver	brain	kidneys
2	2 ml	intraperitoneally	1	1 : 25–50	2+	1+, 1—	neg.	2+
2	,, ,,	,,	3	1 : 25–50	1+, 1—	2+	neg.	neg.
2	,, ,,	,,	7	?	neg.	1+, 1—	neg.	1+, 1—
2	,, ,,	,,	14	?	neg.	neg.	neg.	neg.
4	1 drop	,,	10	1 : 50–100	neg.	neg.	neg.	neg.
3	,, ,,	,,	20	1 : 50–150 lysis	neg.	neg.	neg.	neg.
7	,, ,,	subcutaneous	1	1 : 25–250	neg.	1+, 6—	neg.	neg.
4	,, ,,	,,	2	?	neg.	neg.	neg.	neg.
5	,, ,,	,,	3	1 : 10–25	neg.	neg.	neg.	neg.
3	,, ,,	,,	4	1 : 25–50	neg.	neg.	neg.	neg.
6	,, ,,	,,	5	1 : 50–100	neg.	neg.	neg.	neg.
5	,, ,,	,,	10	1 : 25–50 3 × 1 : 500 lysis	neg.	neg.	neg.	neg.
5	,, ,,	,,	15	1 : 250–1000 lysis	neg.	neg.	neg.	neg.
5	,, ,,	,,	20	1 : 1000 lysis	neg.	neg.	neg.	neg.

been detected by COLLIER (3) in the brains of bats in Batavia. In brain leptospirae occur during a longer period than in blood and liver. The test was repeated after frogs has been inoculated in various ways with virulent Weil leptospirae (incomplete biotype). It was extended by inoculating nearly all the blood and a liver suspension in mice at a varying number of days after the inoculation, together with the agglutinatoricus examination of the blood. During the experiment the frogs sojourned in the tropical room (see Table III).

This examination shows:

a. Only when frogs have been inoculated with many (here 2 ml) virulent pathogenic leptospirae is it possible to find leptospirae in the kidneys up till seven days after the inoculation, so that the excretion of these leptospirae with the urine might be possible in that interval. 47 frogs, inoculated with one drop of virulent leptospirae, did not show any positive kidneys. This indicates that frogs will not excrete leptospirae with the urine, also when a single leptospira (see also under 3) might have succeeded somehow in penetrating into a frog.

b. During the same period as in the kidneys (see under *a*) leptospirae can be detected also in the blood or in the liver, after the longest interval in liver and kidneys. Never were any traced in the brains.

c. Obviously when one drop of a virulent culture has been inoculated, the leptospirae are made innocuous by the existing immunity [1]). If many (here 2 ml; once I succeeded after the injection of one drop of leptospirae) virulent leptospirae are injected, then the immunity may be overcome in the same way as this is the case in a low acquired immunity in guinea-pigs (VAN THIEL (13, 14)).

d. After the injection of one drop of a virulent culture the true agglutination picture changes after a lapse of 10 days after the inoculation at a titer of 1 : 10 till 1 : 50 into that of lysis in the

[1]) This action was studied by inoculating the apathogenic strain Wijnberg of *L. icterohaemorrhagiae* intraperitoneally into frogs and by examining the punctate of the peritoneum in course of time. Up till after 2 hours a few living leptospirae were observed; the greater part, however, was previously agglutinated (already after 15 minutes). The agglutinates soon dispersed into granules. Leucocytes, the number of which increased, ingested dead as well as still living leptospirae.

same way as this happens in actual immune sera. Along herewith the titer rose till 1 : 1000, a value that is never reached in normal frog serum.

It lies nearest to consider this reaction is obviously an anamnestic one). This would explain the absence of leptospirae in blood, liver, kidneys and brain. Against this may tell that this rise of the titer did not occur (for strain Coppée) 15 days after the subcutaneous injection in normal frogs of 1 or 2 ml culture of *L. biflexa*, of $\frac{1}{4}$ or $\frac{1}{2}$ ml normal horse serum and of $\frac{1}{4}$, $\frac{1}{2}$ and 1 ml cholera-typhoid fever vaccine. The antigen chosen as well as the non-intravenous injection might, however, explain this.

e. After the inoculation of virulent pathogenic leptospirae, frogs do not appear to fall ill.

In the next experiment we investigated whether the temperature of the water influences the titer of the serum of frogs after the injection of virulent leptospirae:

6 frogs were injected intraperitoneally with 1.5 ml virulent culture; three of them were placed in water in a room at 21° C., the other three at room temperature (13° C.). The titers of the sera of these frogs, recorded after respectively 15, 20 and 30 days, amounted in the first series (here was lysis) to 1 : 100 (2 ×) and 1 : 250, in the second series to 1 : 50 (2 ×) and 1 : 100. In the first series the titer was thus raised, in the second this was not the case.

So it is evidenced by this experiment that the temperature of the water influences the process going on in the frog.

III. The natural possibility to infect frogs with virulent leptospirae.

This possibility has been studied by bathing frogs in water, contaminated with Weil leptospirae. In table IV a survey of these experiments is offered. The frogs bathed in big bowls of glass, provided with 4 liter of water in a way that they, while sitting on the bottom of the bowl, could reach with the head above the water level. The bathing water was contaminated with the incomplete biotype of *L. icterohaemorrhagiae* isolated from wild rats in Leiden or with the complete biotype of the same species of leptospirae isolated from tame rats of the laboratory of Philips-van Houten Ltd., at Weesp [1]).

[1]) In wild rats in Leiden and its surroundings I did not find the complete biotype. Five or 10 tame rats in Weesp were positive; the strain isolated

Table IV.

Number of frogs	Bath-water: Temperature °C.	Bath-water: Quantity of culture added ml	Duration of the bath (days)	Titer of serum before bathing	Result: Titer of serum 20 days after bathing	Result: Injection of all the blood in mice, immediately after bathing
A. with virulent strain of incomplete biotype of *L. icterohaemorrhagiae.*						
3	17	8	8 [1])	—	1:10, 1:50 (2×)	—
2	30, cooling till 21	18	1	—	1 : 50	20 days after bathing, kidneys negative
2	,,	30	4	2 control frogs 1 : 25 and 1 : 50	1 : 100 (2×) (in one trace of lysis)	—
1 with scarifications on skin						
2	24	6	2×1	1 : 100 (2×)	1 : 50, 1 : 100	—
40 days after operation [2])		6				
1	24	20	2	—	—	negative
B. with virulent strain of complete biotype of *L. icterohaemorrhagiae*						
2	21	40	2	—	—	negative
2	21	38	1	—	—	negative
2	21	38	2	—	—	negative

[1]) At the close of this experiment two guinea-pigs were bathed in this water according to APPELMAN's method. Both died of Weil's disease.

[2]) Blood was collected by cutting the vena femoralis in the left hind-leg (I did not succeed by means of heart puncture); the leg having been ligated above the wound at not too high pressure, the skin was sewn up and the wound provided with collodion. Two of the 5 thus treated frogs were kept alive.

From these experiments we conclude the following. The assumption that Weil leptospirae from strongly contaminated water would

from one of them appeared to be the complete biotype (SCHÜFFNER found 40 % of the tame rats in the same laboratory to be positive).

penetrate easily into frogs is unfounded. If this were actually the case, a distinct lysis would have occurred in their blood like in the experiments recorded in table III; then the leptospirae would probably have disappeared from this water after the frogs had bathed 8 days in it. This was not the case. The only fact that might corroborate the argument of some possibility of penetration of leptospirae into frogs, is supplied by the experiment in which a trace of lysis was observed in one frog 20 days after bathing; the titer of the serum of both frogs was then somewhat raised, when compared with that of the control frogs. This observation, however, bears on water contaminated in such a measure that it is not allowed to apply it to a corresponding condition in nature. But even if frogs abide in heavily contaminated water, then the examination of their blood and kidneys indicates that this does not lead to an infection of the frogs, which would make them play a part in the epidemiology of Weil's disease.

When it is thus possible that a single leptospira might succeed in penetrating into the body of a frog, even then the experiments recorded in table III give certainty that it is made harmless. So frogs behave as immune to an infection with virulent Weil leptospirae.

IV. The congenital immunity of frogs against Weil leptospirae.

For the explaining of the presence of agglutinins against pathogenic leptospirae in the serum of normal frogs two possibilities may be presented.

a. Young frogs are infected in a ditch by pathogenic leptospirae, as a result of which antibodies against these organisms are found in their blood when they are older.

b. In frogs a congenital immunity against Weil leptospirae exists, the nature of which does or does not agree with the acquired immunity, arisen naturally or artificially, in other non immune animal species. Both possibilities require a discussion.

Ad. *a.*

In view of the following facts this possibility may be excluded:

1. The percentage of frogs with positive blood (100 %) with respect to the incomplete biotype of *L. icterohaemorrhagiae* is too high.
2. Frogs from Leiden and its neighbourhood often have agglutinins against the complete biotype of *L. haemorrhagiae* in their blood,

in spite of the local absence of wild rats infected with that biotype.

3. 80 % of the examined frogs had agglutinins against *L. canicola* in their blood, notwithstanding the non-occurrence of this leptospira in ditch water is well established.
4. In normal frogs never any leptospirae were detected, either by microscopical examination of kidneys and brains, or by injection of organs in mice (see under II) as a remnant of a former infection.
5. We have been able to examine a frog which was bred in a small cement pond in a garden in which any contact with rats had practically been excluded. The serum of this frog agglutinated *L. icterohaemorrhagiae* (strain Coppée) and *L. canicola* respectively up to the titers of 1 : 10 and 1 : 4.

If it were true that all frogs, when young, pass through an infection of leptospirae, then it would be very important to know which species of leptospirae, or which biotype of the latter might be held responsible for this; the positive reactions with other species or biotypes should then be considered as co-agglutinations. This question can only be answered by means of CASTELLANI's saturation reaction.

Although SCHÜFFNER and BOHLANDER (12) emphatically state that it is „zwecklos" to examine sera with a titer under 1 : 300, we none the less followed the technics indicated by them (the serum dilutions 1 : 20 up to 1 : 300 could be examined) in order to establish whether the experiment would lead to some conclusion. This has not been successful.

Table V.

Serum of frog after saturation with the strain.

Coppée	Wijnberg	Polly
negative	negative	negative
„	„	„
„	„	„

This negative result, however, does not carry such weight, as the above mentioned five reasons sufficiently prove against a regularly occurring infection of young frogs with virulent leptospirae.

In this argument it has been taken for granted that young frogs could have been infected by any pathogenic leptospira known up till now. Might it not be possible, however, that a at present unknown species of leptospira would play a part, which, after injection of the frog organs in mice, would not induce antibodies, demonstratable by means of the strains available for the present? In such a case, all agglutinations observed in the serum of normal frogs with regard to the known strains should be considered co-agglutinations. This possibility has to be left open, although it seems very improbable, as 1° in all probability co-agglutinations, may it be very low ones, would with the strains used have been observed in the sera of these mice and as 2°, in normal frogs never any leptospirae have been detected microscopically. Neither were they traced in the intestinal contents.

Ad. *b.*

We examined whether the properties of normal frog sera would agree with those of sera of rabbits immunized against pathogenic and apathogenic leptospirae. For that purpose we studied whether any difference would exist between the three sera, 1° by means of precititation of the proteins, and 2° by means of heating of the sera.

Precipitation of the proteins.

The influence of this precipitation on the agglutinin content of the sera was studied as follows.

Centrifuge tubes were provided with constant quantities of immune serum (0.1 ml); increasing quantities of a saturated solution of ammonium sulphate were added; the total volume of each tube was kept on a same level (1 ml) by the addition of sterile tapwater. The tubes, closed with a cock stop, were kept for one hour at 37° C. and subsequently in a relatively cold room. The next morning the precipitated proteins were removed by centrifuging and the agglutination titer (with respect to the incomplete biotype of *L. icterohaemorrhagiae*) of the supernatant liquid was ascertained as customarily (modification of WESTENDORP BOERMA's method (17, p. 70)).

The same was done by means of acetone-alcohol, according to the method described by KAYSER (7). As a ratio of 6 ml acetone to 4 ml alcohol 96 % was the most rapid in inducing the most compact sediment in rabbit serum, this ratio was adopted for the

precipitation. Previously we had ascertained that ammonium sulphate and acetone alcohol did not have any influence on the agglutination of leptospirae.

For the examination of the normal frogs we chose specimina with the highest possible titer of the serum, with respect to strain Coppée (Kantorowicz), *viz.* 1 : 100; the rabbit immune serum was diluted up to the same titer.

The action of the precipitation of the proteins on the disappearance of the agglutinins, respectively lysins from the sera, is found in table VI [1]). Only those experiments have been recorded in which these matters disappear from the serum. As to an explanation of the variability of the occurrence of lysis in the experiments, we remark that this phenomenon occurs merely, when the leptospirae are living; when they are dead only agglutination occurs.

Table VI.

Result of the agglutination-lysis reaction after treatment of rabbit immune serum with ammonium sulphate and acetone-alcohol.

Percentage of saturation	Ammonium sulphate	Acetone — alcohol
a. with rabbit immune serum against *L. biflexa*, strain IX		
28	—	aggl. 1 : 10; lysis up to 1 : 100
30	—	,, ,, ,, ,, ,, ,, 1 : 80
32	—	,, ,, ,, ,, ,, ,, 1 : 50
34	aggl. 1 : 40; lysis up to 1 : 100	,, ,, ,, ,, ,, ,, 1 : 40
36	,, ,, ,, ,, ,, ,, 1 : 90	negative
38	,, ,, ,, ,, ,, ,, 1 : 60	—
40	negative	—
b. with rabbit immune serum against *L. icterohaemorrhagiae* strain Wijnberg		
24	—	aggl. 1 : 10; lysis up to 1 : 100
26	—	,, ,, ,, ,, ,, ,, 1 : 90
28	—	,, ,, ,, ,, ,, ,, 1 : 70
30	—	negative
32	aggl. 1 : 40; lysis up to 1 : 100	—
34	,, 1 : 80	—
36	,, 1 : 40	—
38	negative	—

[1]) Immune sera against leptospirae appeared to behave as sera against species of bacteria, examined by different authors. By this observation we

These reactions were performed also with the serum of frogs, which had been immunized by intraperitoneal injections with *L. biflexa* 1 ×, while abiding in the tropical room. Lysis against the inoculated × strain occurred up till some 1 : 100. The acetone alcohol precipitation was the only one to succeed here; it occurred in a concentration of 28—34 %, thus agreeing with the former experiments.

Contrary to the very definite precipitation of antibodies observed in rabbit sera, was the vague one obtained with the same precipitation methods in normal frog sera. Never did a precipitation occur in frog sera such as described above, showing a titer up to 1 : 100, after about 34 % saturation with acetone alcohol, or after about 38 % saturation with concentrated ammonium sulphate. On the contrary more than once the reaction was wholly negative after saturation with these substances, therefore also in concentrations not inducing precipitation. Opposite to this also a serum was examined, the agglutination of which had disappeared after saturation with 14—16 % and another one after saturation with 10 % acetone alcohol. After saturation with ammonium sulphate in the titrations often pictures were obtained which hardly proved the existence of a positive agglutination.

Heating of the sera.

The titers of the sera were determined without heating and after heating in a water-bath of 56—57° C. during 15, 30, 45 and 60 minutes. Table VII, in which only part of the experiments are recorded, contains the results.

Thus obviously a distinct difference exists between rabbit immune sera and normal frog sera after having been heated. The

do not take side in the open question whether the agglutinins, respectively lysins will occur exclusively in the globulin fraction of the serum (see e.g. Westendorp Boerma contrary to Dunlop). The conception of Zironi and Carlinfanti (18), that the antibodies in leptospiroses are bound at the pseudo-globulin fraction and not, such as it is usually supposed, at the euglobulin, is not well founded, as a convincing chemical difference between euglobulin and pseudoglobulin has not been detected (Westendorp Boerma) and as the conclusion is dependent on the method pursued for separating the proteins. For so far it is true that the part, dissolvable in salt free water of the precipitate obtained by means of acetone alcohol, is the pseudoglobulin fraction of the serum, we have been able to corroborate the conception of both authors.

Table VII.

Source of serum	Agglutination made with strain	Titer of the serum				
		Before heating	After heating during:			
			15 min.	30 min.	45 min.	60 min.
Rabbit antiserum IX	*L. biflexa* IX	1 : 100	1 : 100	1 : 100	1 : 100	1 : 100
Rabbit antiserum Wijnberg	*L. icterohae-morrhagiae* Wijnberg	1 : 100	1 : 100	1 : 100	1 : 100	1 : 100
normal frog	*L. icterohae-morrhagiae* Kantorowicz	1 : 100	1 : 10	1 : 10	1 : 10	1 : 10
,,	,,	1 : 100	1 : 30	1 : 30	1 : 30	1 : 30
,,	,,	1 : 100	1 : 4	1 : 4	1 : 4?	neg.
,,	,,	1 : 30	1 : 4(±)	1 : 4(±)	1 : 4(±)	1 : 4(±)
,,	*L. biflexa* I	1 : 100	1 : 30	1 : 10	1 : 10	1 : 10
,,	,, ,,	1 : 100	1 : 100	1 : 30	1 : 10	neg.
,,	,, ,,	1 : 30	neg.	neg.	neg.	,,
,,	,, IX	1 : 100	,,	,,	,,	,,
,,	,, ,,	1 : 30	,,	,,	,,	,,
,,	,, X	1 : 100	,,	,,	,,	,,
,,	,, ,,	1 : 30	,,	,,	,,	,,

former are thermostabile at the temperature of the experiment, the latter are not [1]). The agglutination as well as the lysis are kept up. Immune sera of frogs (Table VIII) obviously take up a middle position.

It thus follows from the experiments, bearing ad *b*, that the properties of the antibodies in normal frog serum are not the same as those in rabbit immune serum (It is better not to speak of ,,natural immunity'', as this may bear also on the immunity that arises after an infection and can thus be opposed to an active or passive immunisation). By showing this difference, more particularly in the effect of heating, normal frog serum behaves as normal sera of other animals with respect to different bacterial species do. Bordet, Rodet, Negré, Landsteiner and Reich, Trazek (quoted by Bordet (2)), did observe that normal agglutinins

[1]) By heating the above mentioned retiform type of agglutination initially changes into a more threadlike type; the following morning the more or less compact threads are balled together to thick clods.

tolerate heating in a lesser degree, so that they seem „plus fragile" than agglutinins of immune sera. As immune sera show for their antigens a much more powerful affinity, a decrease of their activity (the agglutinating capacity of immune sera is lowered as well) does not occur so definitely as a weakening in the energy of the agglutinins of normal sera. According to CANTACUZÈNE and NOGUCHI (quoted by BORDET) the antibodies of cold blooded animals are even particularly susceptible for heating, so that their activity would have diminished very distinctly already at about 50° C. As, however, according to BORDET, normal and immune sera of warm blooded animals can stand heating at 55° C. very well — the agglutinating capacity of normal serum of guinea-pig, goat, dog, horses and chicken being weakened after heating for half an hour at 61 or 62° C. — the origin of the serum has a great influence on the temperature at which agglutinins are destroyed. This would be bound up with the fact that not all sera coagulate at exactly the same temperature.

But though normal agglutinins and those of immune sera do not respond in exactly the same manner to heating, still BORDET does not ascribe the greater susceptibility of the former to the coming into play of another principle, but to their lesser strength. At any rate he does not consider the difference large enough for the assuming of a sharp distinction between normal and immune antibodies. Both principles would belong to a same group of phenomena. Certainty with regard to this, however, can not be attained at, as long as the manner of formation of normal antibodies can only be guessed at (BORDET). Perhaps normal products of metabolism of the frog are allied to agglutinins against leptospirae (in accordance with MÜLLER (8)), so that the phenomenon of agglutination can only act, thanks to this affinity.

The result of the experiment recorded in Table VIII tells as well in favour of the conception of BORDET of the non-existence of a sharp distinction between normal and immune antibodies.

Five frogs were immunized, while abiding in the tropical room, by weekly intraperitoneal injections of successively ½, 1, 2, 4 and 8 ml leptospiral culture. The serum collected after one week has been examined according to the above described method of heating. Notwithstanding these frogs were immunized similarly and simultaneously, the results were not uniform. In the first place it is fairly certain that no, or hardly any immunization has taken place

Table VIII.

Origin of the serum	Agglutination performed with strain	Titer of the serum				
		Before heating	after heating during:			
			15 min.	30 min.	45 min.	60 min.
frogs 1	*L. biflexa* IX	1 : 300 (ly)	1 : 100 (ly)	1 : 100 (ly)	1 : 30 (ly)	1 : 30 (ly)
immu- 2	,, ,,	1 : 300 (ag)	1 : 200 (ag)	1 : 10 (ag)	neg.	neg.
nized 3	,, ,,	1 : 100 (ag)	1 : 30 (ag)	1 : 20 (ag)	neg.	neg.
with 4	,, ,,	1 : 100 (ag)	1 : 20 (ag)	neg.	neg.	neg.
L. biflexa 5	,, ,,	1 : 300 (agly)	1 : 300 (agly)	1 : 300 (agly)	1 : 200 (agly)	1 : 100 (agly)
strain IX.						

in the frogs 2, 3 and 4. In the other frogs this has actually been the case, but the antibody behaved here otherwise than in the rabbit.

If it is actually so, that normal sera are more sensitive than immune sera, then this can find its expression also in the different reactions with respect to the saturation with ammonium sulphate and acetone alcohol. The mechanism of the congenital immunity of frogs needs to be made a subject of continued research.

Summary.

1. A high percentage of frog sera (of *Rana esculenta* and *Rana fusca*) reacts positively with respect to many pathogenic and apathogenic leptospira-strains; this reaction is highest (100 %) with respect to the incomplete biotype of *L. icterohaemorrhagiae*.
2. Under natural conditions frogs do not excrete leptospirae with the urine and neither is this the case, when a single leptospira has somehow succeeded in penetrating into them. This penetration does not easily take place.
3. By the inoculation of many virulent leptospirae it is possible to break the congenital immunity. Up till 3 days after the inoculation leptospirae can be detected in the blood, up till 7 days in the liver and kidneys. The frogs did not appear to fall ill.
4. Frogs do not play a part in the epidemiology of Weil's disease.
5. In normal frog sera precipitation of the agglutinins by means of ammonium sulphate and acetone alcohol could not be observed so definitely as in immune sera. Normal frog serum is thermostabile at 56—57° C., immune serum thermolabile at the same temperature. These differences are explained, in accordance

with BORDET, by the lesser strength of the antibodies in normal sera.

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AVIDITY

A FACTOR TO BE CONSIDERED IN THE INTERPRETING OF THE AGGLUTINATION-INHIBITION TEST FOR INFLUENZA A VIRUSES

by

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A. I.

Our laboratory strain PR. 8 shows a four times lower agglutination titer for human O erythrocytes than for chick red cells. (Table I). Thus for an agglutination-inhibition test, using the same multiple of the agglutination unit, the fourfold of the amount of virus must be used, if this test is carried out with human erythrocytes in stead of with chick red cells. This being the case, four times as much serum should be expected to be needed for human cells.

Table I.

The difference between the end dilution for human red cells and chick cells.

virus strain	Human O cells 1/4 %	Chick red cells 1/4 %
PR. 8 ($F_{198}M_{593}E_{258}$)	1/3840	1/15360

Our standard technic for agglutination-inhibition tests is as follows:

Standard dilutions of the sera are made $^1/_4$; $^1/_6$; $^1/_8$; $^1/_{12}$ etc, and in each tube unto 1 ml serum dilution 1 ml of a virus suspension containing 12 agglutination units per ml is added. After storing at room temperature for 30 minutes, unto every tube 1 ml of a red cell suspension of $^3/_4$ % is added. After 2 hours at room temperature the racks are put in the refrigerator at + 4° C. (39° F.) and allowed to stand for 10 hours or more. The end dilutions of the sera containing 4 agglutination units of virus and $^1/_4$ % red cells per ml, are $^1/_{12}$; $^1/_{18}$; $^1/_{24}$; $^1/_{36}$ etc.

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As diluent salt solution 0.9 % in distilled water is used. The inhibition values read are the intermediates between the values for total agglutination and for none at all; allowing the marks +, ++, +++ for partial agglutination.

As controls we add one tube with 1 ml serum dilution $^1/_4$, salt solution 1 ml and red cell suspension $^3/_4$ % 1 ml.

For every virus suspension used one rack with dilutions (as above) is made. One virus agglutination unit per ml is the last tube with complete agglutination.

The inhibition value read, multiplied by the number of agglutination units of the virus used, is the inhibition titer of the serum.

The test was carried out with 4 agglutination units. All tests in one table were performed with the same virus suspensions, all at one time.

The antisera were made by injecting 5 ml allantoic fluid intraperitoneally into rabbits (FRANCIS (3)).

As table II shows, the inhibition-titer of serum, when using human O erythrocytes, is higher than was expected.

Table II.

The difference of the values obtained if human red cells in stead of chick erythrocytes are used in inhibition tests with the PR. 8 strain.

Serum	Chick red cells	Human O erythrocytes	
		found	calculation
II anti PR. 8	1/30720	1/15360	1/7680
III anti PR. 8	1/21600	1/10800	1/5400
XI anti PR. 8	1/23040	1/11520	1/5760

WHITMAN (6) already mentioned, that agglutination-inhibition tests carried out with human erythrocytes are more sensitive than when chick cells are used. Every time we tried to eliminate some possible faults, the same results were obtained.

A. II.

A possible solution of this problem could be, that not the same particles of the virus, or components of the virus particles are causing the agglutination of both human erythrocytes and chick red cells. The avidity of the serum antibodies for both kinds of particles might differ in that case.

The first point to consider is: is there any reason as to the existence of such different particles?

It is already known, that amniotic- and allantoic fluids containing newly isolated influenza viruses, agglutinate more readily human erythrocytes than chick red cells. In the course of passing

from egg to egg, the fluids will agglutinate more and more easily chick cells as compared to human- or guinea pig cells. This is one of the phenomena showing that the virus is passing from one phase to another (from O to D as described by BURNET (1)). After 5 or 6 passages, however, there is still a significant ability to agglutinate human cells as compared with chick cells (Table III).

Table III.

Comparison of a newly isolated strain with a laboratory strain as to its ability to agglutinate human O erythrocytes and chick red cells.

Virus strain	Dilution of the allantoic fluid	
	Human O cells 1/4 %	Chick red cells 1/4 %
virus H. 5th egg passage	1/5120	1/5120
virus H. 9th egg passage	1/5120	1/7680
Lab. strain PR. 8 ($F_{198}M_{593}E_{257}$)	1/2560	1/10240

In fact it may be possible, that not the same particles of the virus, or components of the virus particles, are responsible for the agglutination of both kinds of red cells, the mutual ratio of these particles changing during the passages from egg to egg. We have tried to separate those factors but failed up till this moment.

An example: If a PR. 8 virus suspension had been treated with human red cells it was impossible to recover a fluid able to agglutinate chick cells (either at room temperature (15° C. = 60° F.) or at 4° C. = 39° F.). As sheep red cells are also able to adsorb influenza virus, even when they will not be agglutinated by this virus, the test mentioned does not give us an answer to the question whether the same or different particles are responsible for the agglutination of both kinds of erythrocytes.

A. III.

If we suppose, that different particles or components of the virus bodies are causing the agglutination of human red cells and of chick erythrocytes, the sera may also have a different avidity to those components or particles. To test this supposition we altered the technic of the agglutination-inhibition test:

The virus suspension was divided in four equal parts, added at intervals of 10 minutes to the serum dilutions.

After adding the first portion of the virus there are too few virus particles with respect to the antibodies. All virus particles of either kind are bound to the antibodies. Afterwards a second portion of the virus is added. First all human factors are bound. There remain still antibodies for the chick factors, which are also bound. This repeats itself and in this way either kind of particles gets a same chance of getting hold of the antibodies. In the standard method however, the whole portion of the virus being at once added to the serum, the particles or components responsible for agglutination of human erythrocytes, owing to a greater avidity, will take away relatively more of the antibodies.

It is evident, that if our assumption is right, in the fractionated method more serum will be needed to inhibit the same quantity of „human" factor as in the standard method.

The results were conform our expectation. Table IV shows the figures obtained with both methods. As the results with the fractionated method correlate closely with the calculated figures, we assume that an avidity factor of the serum is the actual reason for the above mentioned disparity. Of course the problem may be not as simple as it is presented here. There may be more kinds of virus particles or components of those, each of them with their own avidity factor in the serum.

Table IV.

The demonstration of the avidity factor by fractionated adding of the virus in agglutination-inhibition tests with the PR. 8 strain.

Serum	Chick cells		Human O erythrocytes		
	4 agglut. units	16 agglut. units	4 aggl. units = 16 chick U.		
			found	calculat.	fract.
I anti PR. 8	1/3600	1/3600	1/1800	1/900	1/900
II anti PR. 8	1/14400	1/14400	1/8400	1/3600	1/3600
VII anti H	1/9600	1/9600	1/7200	1/2400	1/2700
VIII anti H	1/7200	1/7200	1/6000	1/1800	1/2100

When we assume that the sera have a greater avidity for the „human" cell factor, than for the „chicken cell" factor we may expect that fractionated adding of virus will have no influence on the results of tests carried out with chick cells. In both instances — standard method or fractionated method — the „human factor"

will be saturated with antibodies before the chick factor is bound.

The results of our experiments were quite in agreement with this expectation.

B. I.

A second point to consider: an agglutination unit is not an absolute gauge for virus concentration. As in agglutination-inhibition tests as virus unit the agglutination unit is used, the results of such tests are obviously only of a relative value.

Under A. II we already called attention to the fact, that different virus suspensions vary in their proportion between the dilutions representing one agglutination unit for human erythrocytes and one for chick red cells (Table III). Of the PR. 8 strain mentioned in table III one agglutination unit for human cells must be diluted 4 times to reach one agglutination unit for chick erythrocytes. On the other hand the 5th passage of strain H uses the same dilution for one agglutination unit with either suspension of red cells.

It is obvious therefore, that if strain H 5th passage is compared with strain PR. 8 in inhibition tests, the results of tests carried out with chick cells cannot be the same as those obtained with human erythrocytes.

B. II.

In this example we will naturally meet the same avidity factor as we encountered in part A. The influence on the values for the 5th passage of strain H will be weak.

The ratio ,,human cell''-,,chicken cell'' factor being 1 : 1 we use the same quantity of virus in inhibition tests with both cells. Even then, less serum is needed to inhibit the ,,human'' factor than to inhibit the ,,chicken'' factor as in the former case, part of the ,,chicken'' factor owing to its lower avidity will remain free.

The influence of the avidity on the results of strain PR. 8 will be more important. In this case, a lot of ,,chicken cell'' factor remaining unbound, the avidity factor strongly diminishes the differences in values expected if the inhibition tests were carried out with human erythrocytes instead of with chick red cells. But even so, it remains obvious, that differences in the results exist.

Comparision between the antigenic composition of influenza strains in the agglutination-inhibition test is without value if both strains are not in the same O-D phase.

In Table V (sera II and III) this fact is demonstrated.

Table V.

Serum	Chick cells		Human O erythrocytes			
	PR. 8	H. 5th pass.	PR. 8	(PR. 8 calc.)	H	H(calc.)
II anti PR. 8	1/30720	1/15360	1/15360	1/7680	1/23040	1/15360
III anti PR. 8	1/21600	1/10800	1/10800	1/5400	1/14400	1/10800
VII anti H	1/14400	1/7200	1/10800	1/3600	1/10800	1/7200
VIII anti H	1/15360	1/7680	1/11520	1/3840	1/11520	1/7680

C.

In Table V a third point is to be considered: do all antisera have an equal avidity factor?

The answer must be in the negative, for the sera prepared by injecting a newly isolated strain intraperitoneally into rabbits, show a greater avidity factor, than sera prepared in the same way with the PR. 8 laboratory strain (Table V sera VII and VIII).

The values in the tables are taken out of a great many tests carried out in the same way.

Discussion.

Table V shows us clearly, that there is a disparity in the results of agglutination-inhibition tests carried out with the same virus when human red cells or when chick erythrocytes are used as indicators.

In the case of human erythrocytes the amount of serum necessary to inhibit the haemagglutination is in proportion to the virus-concentration used smaller than when chick red cells are used. The solution for this problem is given in table IV. By means of fractionated adding of the virus nearly equal results are obtained with both kinds of erythrocytes.

This being the case, we may conclude that an avidity factor is the reason for the noted disparity. Theoretically we must assume that there are two kinds of virus particles in our virus-suspensions, one responsible for the agglutination of human erythrocytes and one for the agglutination of chick cells. Naturally, there may be more kinds of virus particles.

Also it may be possible, that not particles, but components of

the virus-bodies are actually causing the agglutination of the various cell suspensions.

In the tables shown it is clearly demonstrated, that the avidity-factor has not always the same value in different sera. In specific rabbit anti-sera prepared by us the greatest avidity values were found for sera prepared with newly isolated influenza strains.

This avidity phenomenon gives rise to difficulties in the using of agglutination-inhibition tests for the study of the antigenic structure of influenza strains. These difficulties can be avoided if strains are compared which are in the same O till D phase. In other words: strains that have a comparable ratio between the amount of particles or components causing agglutination of human cells and components or particles responsible for the agglutination of chick erythrocytes. The necessity is to be stressed that in comparing two virus strains, the antigenic disparity found in tests carried out with human erythrocytes be of the same order as that found in tests carried out with chick red cells.

The avidity problem is not new. In the titrations between diphtheriatoxine and -antitoxine it is a since long accepted feature (2). Hirst (4) described only a short time ago a difference in avidity between sera made against a mice passed and a egg passed strain of the same influenza virus. He also concluded, that an avidity-factor must have been the reason for the discrepancy noted.

The vagaries encountered by Stuart Harris and Miller (5) may also have been due to the avidity factor. The easiest solution for this problem should be to repeat their titrations with human red cells. Their newly isolated virus may have had a great many „human" particles. The use of fractionated adding is a very complicated technic with great possibilities for faults. It is not advisable for routine tests.

We did not find any differences in agglutinability of the erythrocytes of our chickens such as Stuart Harris observed in his ones. This may be due to the fact that we used only the erythrocytes of four fowls of one inbred white leghorn strain.

Summary.

There is a discrepancy between the results of inhibition tests carried out with human erythrocytes and the values found if chick red cells are used.

It is shown, that if an avidity factor is introduced, the disparity in results can be explained. It is possible to eliminate the avidity factor by means of fractionated adding of the virus suspension.

The way to avoid possible faults in the future is discussed.

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LA DÉTERMINATION SÉROLOGIQUE DE LA POLIOMYÉLITE ASYMPTOMATIQUE DANS LA FAMILLE

par

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Comme on s'y attendait l'adaptation de quelques souches du virus de la poliomyélite humaine à des souris a permis d'obtenir sur l'immunité de la population à la poliomyélite des données plus nombreuses que les coûteuses épreuves de neutralisation sur les singes faites jusqu'ici. Tandis que d'après HARFORD et BRONFENBRENNER (5) et d'autres, les résultats des épreuves de neutralisation sur des souris faisaient supposer que dans le cours de la poliomyélite — bien que ce ne soit pas constant — il peut se former des anticorps qui neutralisent la souche Lansing, ARMSTRONG (1), TURNER et YOUNG (13), BROWN et FRANCIS (2) et d'autres encore ont montré que 65 à 75 pour cent des sérums humains normaux peuvent également neutraliser cette souche. Aussi, la signification qu'il faut accorder à la présence de ces anticorps n'est-elle pas encore clairement établie. Comme il se trouve que le pourcentage de sérums, dans lesquels on peut déceler des anticorps contre la souche Lansing ou contre d'autres souches murines et d'origine humaine est un pourcentage très élevé, on suppose que le virus de la poliomyélite est très répandu chez la population de régions endémiques, et constitue souvent une infection asymptomatique. OLITSKY et FINDLAY (11) soulignent le fait que cette opinion se trouve seulement étayée si les anticorps ne se forment que comme conséquence spécifique d'une infection poliomyélitique, éventuellement asymptomatique. Plusieurs auteurs, en défendant la théorie de maturation, sont d'avis que les anticorps ne sont pas spécifiques.

A plusieurs reprises on a pu, chez des porteurs de germes restés sains déceler le virus de la poliomyélite, notamment dans les selles. Vraisemblablement une immunité se produit chez ces personnes, tandis que des anticorps apparaissent dans leur sérum. MELNICK et HORSTMANN (10) ont démontré que des chimpanzés infectés expérimentalement et dans lesquels une poliomyélite cliniquement perceptible ne se développe pas, s'immunisent en effet, et qu'il se forme des anticorps.

Nous nous sommes occupés à l'examen des malades atteints de poliomyélite, et des membres de leurs familles, concernant la présence d'anticorps neutralisant la souche Lansing. Notre communication provisoire suivante pourrait faire conclure que chez l'homme également une infection asymptomatique peut faire apparaître des anticorps neutralisants.

Dans la famille L un garçon âgé de 11 ans est admis souffrant de poliomyélite, forme paralytique légère. Dans la phase aiguë le sérum du malade, de ses parents, d'une soeur âgée de 12 ans et d'un frère âgé de 8 ans est examiné sur la présence des anticorps neutralisant la souche Lansing. Dix semaines après, le malade étant presque guéri, nous avons examiné un nouvel échantillon de sérum des mêmes individus.

Comme on songeait aussi à l'éventualité d'une chorioméningite lymphocytaire, maladie dont le syndrome peut être semblable à celui de l'encéphalomyélite (VERLINDE, VAN DER WERFF et BRIËT (14)), on a fait des titrages d'anticorps contre le virus de la chorioméningite lymphocytaire aussi.

Pour faire l'épreuve de neutralisation on a mélangé à parties égales d'une part: des suspensions de virus à concentrations décroissantes (cerveau de souris infectées par voie cérébrale) avec du sérum non dilué, d'autre part des mêmes suspensions de virus avec de l'eau physiologique. Après avoir incubé les mélanges pendant une heure on a inoculé, par voie cérébrale, 4 souris. Le tableau I reproduit les résultats de cette épreuve de neutralisation.

On considère qu'un sérum contient une quantité significative d'anticorps quand il neutralise 100 ou plus de 100 doses cérébrales mortelles pour des souris. De sorte que chez toutes les personnes figurant dans le tableau I, le pouvoir neutralisant à l'égard du virus de la chorioméningite lymphocytaire est insignifiant. Par contre la souche Lansing du virus de la poliomyélite se trouve neutralisée par le sérum de la phase aiguë comme par celui de la phase de

Tableau I.

		Concentrations finales du virus							
		Souche Lansing				Chorioméningite			
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Premier sérum	Contrôle	4/4	3/4	2/4	0/4	4/4	2/4	2/4	0/4
	Malade	0/4	0/4	0/4	0/4	3/4	2/4	0/4	0/4
	Père	2/4	2/4	0/4	0/4	3/4	2/4	2/4	0/4
	Mère	0/4	0/4	0/4	0/4	3/4	2/4	2/4	0/4
	Soeur	3/4	2/4	2/4	0/4	2/4	2/4	0/4	0/4
	Frère	3/4	4/4	0/4	0/4	2/4	2/4	0/4	0/4
Deuxième sérum	Contrôle	3/4	4/4	2/4	0/4	4/4	4/4	2/4	0/4
	Malade	0/4	0/4	0/4	0/4	4/4	4/4	2/4	0/4
	Père	4/4	0/4	0/4	0/4	3/4	3/4	2/4	0/4
	Mère	0/4	0/4	0/4	0/4	4/4	4/4	3/4	1/4
	Soeur	0/4	0/4	0/4	0/4	4/4	4/4	4/4	2/4
	Frère	3/4	2/4	2/4	0/4	2/4	2/4	2/4	0/4

Le dénominateur indique le nombre de souris inoculées, le numérateur le nombre de souris mortes.

convalescence chez le malade aussi bien que chez la mère. Les trois autres sérums premiers n'ont pas un pouvoir neutralisant. Le deuxième sérum du père et surtout de la soeur, a un pouvoir neutralisant sensiblement plus élevé, bien que ni l'un ni l'autre n'ait été malade.

A l'égard du titre élevé d'anticorps dans le sérum du malade pendant la phase aiguë il y a lieu de se demander si dans cette phase les anticorps sont une réaction à l'infection ou s'ils existaient déjà. Il en est de même du premier sérum de la mère: y a-t-il chez elle en même temps ou presque en même temps que chez le malade une infection asymptomatique, ou les anticorps étaient-ils déjà présents avant? On ne peut donner de réponse définitive à ces questions. Il faut faire remarquer que la possibilité de la formation d'anticorps pendant la phase aiguë ne peut être exclue. Il est vrai que le sérum de la phase aiguë a été obtenu le deuxième jour de la phase paralytique, mais après une période prodromique d'au moins de 8 jours. Si l'on tient compte aussi de la période d'incubation, il doit y avoir eu assez de temps pour permettre la formation d'anticorps.

Si les anticorps ne résultent pas de celle-ci, mais d'une infection

antérieure et asymptomatique, le malade n'aurait-il pas dû être immunisé? Bien que la présence d'anticorps n'entraine pas nécessairement l'immunité, on peut dire qu'une immunité en formation s'accompagne de la production d'anticorps. Il faut toutefois tenir compte de ceci: il se présente des souches de virus d'une autre structure antigène, de sorte qu'une première infection ne confère pas nécessairement l'immunité à une deuxième infection, lorsque les deux souches de virus n'ont aucun rapport antigène. Le fait que, dans le cours de la maladie de l'enfant, il se soit formé des anticorps à la souche Lansing dans le sérum du père et de la soeur, pourrait plaider en faveur d'une infection de la famille par un virus, dont la structure antigène est étroitement apparentée à celle de la souche Lansing. Si l'on tient compte de ces considérations, il n'est pas illogique d'admettre que les anticorps de la phase aiguë se sont formés au cours de cette infection. La hausse du titre d'anticorps chez le père et chez la soeur entre le début et le fin de la maladie chez le garçon, fait supposer que tous deux ont été exposés à une infection asymptomatique par un virus poliomyélitique de structure antigène analogue à celle de la souche Lansing.

Le deuxième sérum du malade et de sa mère a été examiné en vue d'y déceler la présence d'anticorps à deux souches de structure antigène autre que celle de la souche Lansing, notamment la souche SK de Jungeblut et Sanders (9) et la souche MM de Jungeblut et Dalldorf (8), considérées toutes les deux comme étant d'origine humaine. L'épreuve de neutralisation intracérébrale n'a pas pu déceler la présence d'anticorps contre ces souches.

Il est tout indiqué de conclure que le malade qui était à la maison pendant la période préparalytique, a infecté le père et la soeur. Il se peut toutefois que la mère, dans le sérum de laquelle on a pu, dès les premiers jours de la maladie, déceler des anticorps contre la souche Lansing, ait été porteur de virus et par conséquent source de contagion. Ni l'un ni l'autre n'a été en rapport avec un malade atteint de poliomyélite cliniquement établie.

Comme l'examen sérologique révèle une infection de famille, il eût été intéressant de suivre le cours de l'infection. N'ayant pas de singes il nous a malheureusement été impossible de faire un examen sur la présence du virus.

Taylor et Amoss (12) déjà ont montré le virus de la poliomyélite dans les sécrétions naso-pharyngées d'un membre de famille qui à été en contact avec un malade, et qui lui-même est atteint de

poliomyélite cinq jours après. D'après Howe, Bodian et Wenner(7) et Horstmann, Melnick et Wenner (6) ce n'est généralement que pendant les trois premiers jours de la maladie que le virus peut être isolé du pharynx. Casey (3) se basant sur des recherches épidémiologiques arrive à la conclusion que la plupart des malades de poliomyélite sont contagieux pendant les trois jours avant et les trois jours après le début de la maladie. Son opinion se trouve étayée par des recherches expérimentales de Gordon, Schabel, Casey et Fishbein (4) sur la présence du virus de la poliomyélite chez des enfants qui ont été en contact direct avec des malades. Aussi les derniers auteurs considèrent le pharynx comme la voie principale par laquelle — en tout cas dans la phase contagieuse — le virus est excrété et comme la principale porte d'entrée. Par conséquent d'après lui la contagion par contact se fait par voie naso-pharyngée. Cela n'exclut pas les matières fécales comme source de contagion indirecte.

Chez la famille qui a été le sujet de notre étude et qui vit dans d'excellentes conditions hygiéniques une contamination par les matières fécales nous semble peu probable. Si en effet le père et la soeur ont été infectés par le malade (ou par la mère) la contamination par voie naso-pharyngée nous semble la plus vraisemblable.

Un demi an plus tard, en avril et mai 1948, dans un village ou se présentaient quelques cas de poliomyélite légère, nous avons examiné le sérum de la phase aiguë et le sérum de la phase de convalescence de trois malades et de 4 personnes de contact dans trois familles. Les épreuves de neutralisation faient avec les trois souches du virus poliomyélitique (Lansing, SK et MM) et avec le virus de la chorioméningite lymphocytaire. La souche Lansing et le virus de la chorioméningite lymphocytaire ont été injectés par voie cérébrale, les souches SK et MM, à cause de leur haute virulence, par voie intrapéritonéale. La dose cérébrale des mélanges de virus et sérum était 0,02 cc, la dose intrapéritonéale était 0,1 cc. Pour controler la virulence des souches de virus dans les conditions données on a injecté les mêmes dilutions de virus mélangées avec du sérum normal de cheval. Les résultats sont figurés dans la tableau II, dans lequel on n'a indiqué que les doses mortelles des mélanges de virus et sérum.

Tableau II.

Souches de virus	Lansing		Choriomé-ningite		SK		MM	
Premier (P) et deuxième (D) sérum	P	D	P	D	P	D	P	D
Contrôle (sérum de cheval	10^{-3}	10^{-3}	10^{-2}	10^{-3}	10^{-6}	10^{-6}	10^{-3}	10^{-4}
Malade O.	$<10^{-1}$	$<10^{-1}$	10^{-1}	10^{-3}	10^{-6}	10^{-6}	10^{-4}	10^{-5}
Père O.	10^{-1}		10^{-1}		10^{-6}		10^{-5}	
Mère O.	$<10^{-1}$		10^{-1}		10^{-6}		10^{-5}	
Soeur O.	10^{-3}		10^{-1}		10^{-6}		10^{-4}	
Malade J.V.	10^{-3}	10^{-3}	10^{-2}	10^{-2}	10^{-6}	10^{-6}	10^{-5}	10^{-5}
Malade H.V. (forme inapparente)	$<10^{-1}$	$<10^{-1}$	10^{-1}	10^{-3}	10^{-5}	10^{-5}	10^{-4}	10^{-5}
Malade T.	10^{-2}	$<10^{-1}$	10^{-1}	10^{-3}	10^{-5}	10^{-5}	10^{-3}	10^{-5}

La famille O. ne fut examinée qu'incomplètement, une deuxième ponction chez les personnes normales ayant été refusée. Le sérum de la phase aiguë du malade, son sérum de convalescence et le premier sérum de la mère avaient un pouvoir neutralisant complet à l'égard de la souche Lansing, tandis que le sérum du père neutralisait cette souche partiellement.

Une source de contagion commune pour cette famille et qui n'a pas atteint la soeur nous semble acceptable. On ne peut pas faire des conclusions concernant une infection éventuelle de la soeur par le malade ou par son père ou sa mère, parce qu'on n'a pas pu faire une deuxième examination sérologique.

Dans la famille V., une fillette âgée de 3 ans (J.V.) était malade pendant 17 jours au moment de la première prise de sérum. Elle présentait une parésie légère du bras gauche. Son frère, âgé de 4 ans (H.V.) se plaignait simultanément de douleur du cou et dans son liquide céphalo-rhachidien on trouvait 610 cellules par mm cube, principalement des lymphocytes, tandis que les réactions de Nonne et de Pandy étaient positives. Les deux sérums du garçon neutralisaient complètement la souche Lansing, ainsi à l'opposé des sérums de la fillette. Ici aussi, une source de contagion commune semble probable, mais singulièrement seul le sérum du garçon avait un pouvoir neutralisant contre la souche Lansing. Ou le virus

étiologique n'est pas de structure antigène analogue à celle de la souche Lansing, dans quel cas on se demande si les anticorps se sont formés comme réponse à une infection asymptomatique antérieure, ou bien chez la fillette des anticorps ne se sont pas formées pour raison inconnue.

Le malade T, un voisin de la famille O, était simultanément atteint d'une méningite lymphocytaire. Tandis que son sérum de la phase aiguë ne neutralisait pas évidemment la souche Lansing, le sérum de convalescent neutralisait cette souche complètement. La chorioméningite lymphocytaire pouvait être exclue à cause de l'examen sérologique.

Bien qu'on ne puisse pas tirer des conclusions définitives de cette communication provisoire, de l'examen de la famille L et du malade T il parait que les anticorps ne se forment que comme conséquence spécifique d'une infection, éventuellement asymptomatique, par un virus poliomyélitique d'une structure antigène apparentée à celle de la souche Lansing, tandis qu'on a obtenu dans la famille L une indication concernant la possibilité d'une infection par contact direct dans la famille.

On n'a pas pu déceler d'anticorps neutralisant les souches SK et MM. Nous sommes spécialement frappés de la neutralisation partielle du virus de la chorioméningite par le sérum de la phase aiguë des malades atteints de poliomyélite, tandis que ce pouvoir neutralisant a disparu dans le sérum de convalescent.

Résumé.

Dans 4 familles où se trouvait un malade atteint de poliomyélite on a examiné les sérums de la phase aiguë et de la phase de convalescence des malades et des autres membres de famille pour chercher la présence d'anticorps neutralisant trois souches du virus poliomyélitique (Lansing, SK et MM) et le virus de la chorioméningite lymphocytaire. Dans deux familles au moins, les recherches indiquent que les anticorps contre la souche Lansing ne se forment que comme conséquence spécifique d'une infection, éventuellement asymptomatique, par un virus poliomyélitique d'une structure antigène apparentée à celle de la souche Lansing. Dans une famille le sérum de la phase aiguë d'un malade et le sérum de sa mère, contenaient des anticorps tandis que le sérum de son père et d'une soeur ne neutralisait pas la souche Lansing. Dix

semaines après, les sérums du père et de la soeur avaient obtenu un pouvoir neutralisant contre cette souche, bien que ni l'un ni l'autre n'aient été malades. Ceci pourrait indiquer la possibilité d'une infection par contact direct dans la famille.

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(From the Laboratory of the Public Health Service, Curaçao N.W.I.).

THE EPIDEMIOLOGY OF SHIGELLOSIS (BACILLARY DYSENTERY) ON CURAÇAO

by

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(Received May 15, 1948)

The island of Curaçao is one of the Lesser Antilles and is situated near the North coast of Venezuela (Lat. ± 12° N., Long. ± 69° W.).

Its surface amounts to 173 sq. miles. The mean annual temperature is ca. 80° F. and varies between ca. 78° F. in January or February and ca. 82° F. in September or October.

The island is completely devoid of lakes, streams and canals. Rainfall is very variable but may be expected in October, November, December and January. Even in these months downfall can be scanty and insufficient for economical needs. The rain usually falls in heavy showers of short duration and as the rocky soil cannot quickly absorb such great quantities of water, a big part is lost into the sea (JANSEN (2)). The climate of the island can be best compared with that of a tropical steppe, refreshed by the North-Eastern tradewind.

The population increased from 65.231 on Dec. 31, 1938 to 88.323 on Dec. 31, 1946. According to expert estimation about three fourths of these numbers were born in the island as descendants of European settlers and of African negroes. With the exception of a small number of whites they are coloured in all shades from very light brown to black. The much varied „foreign" group (± 40 nationalities) comprises 5 to 6 thousands European Dutch. The immigration of comparatively large numbers of young adults of both sexes who after a shorter or longer stay will return to their home country accounts for remarkable deviations from the age groups of the standard population of KÖRÖSY (WINCKEL (10)). Industry (oilrefinery), mining (phosphate), trade and traffic are the principal means of subsistance.



Fig. 1. Map of Curaçao (With permission of Tjeenk Willink's Uitg. Mij.).



Fig. 2. Willemstad and part of the Oilrefinery. X Fort Nassau.

Until the fall of 1939 the official opinion prevailed that Curaçao was practically free of bacillary dysentery. Observed cases of dysentery were supposed to be caused by *Entamoeba histolytica* and diarrhea without blood and mucus was recorded as ,,enteritis'' or ,,gastro-enteritis''. This situation is common in all tropical places where diagnostic facilities are poor (WENYON (9)).

Soon after the arrival of a bacteriologist in 1939 positive isolations of Shigellae proved that bacillary dysentery was the most frequently occurring notifiable contagious disease and responsible for the death of many infants and young children (POT, VAN RAALTE and VAN DER SAR (4)).

With regard to the species and types of the causative agents, of 1387 strains cultured between Sept. 1939 and Jan. 1946 78.8 % belonged to the species *Shigella paradysenteriae*, 13.7 % were Sonne strains, while *Shigella ambigua* was represented in 7.4 % of all cases. *Shigella dysenteriae* was isolated twice in 1943.

An analysis of *Shigella paradysenteriae* in 1942 by means of sera from the Standards Laboratory (Medical Research Council, England) and a second one in 1945—46 with the type sera of Lederly Laboratories revealed that types ,,W'' (Flexner II) and ,,Z'' (Flexner III) are the most common. Type ,,Newcastle-88'' (Flexner VI) is not very rare and is represented by all its biochemical varieties; by far the bigger part, however, belongs to the ,,Type 88'' of BOYD (POT (6)).

The present paper will give a discussion on the spreading of bacteriologically confirmed cases over the months of the period between September 1939 and July 1947. The Public Health Regulations of Curaçao order only those cases of supposed dysentery to be recorded in which this diagnosis has been confirmed in the Laboratory of the Public Health Service. In this rather long time all notifications passed the hands of the author, who was in charge of the bacteriological examinations.

The recorded cases are of course only a minority of the actual number of infections. A certain, unknown, part is being missed due to imperfections of bacteriological technique. Another part does not come under the care of a doctor and if so, does not have bacteriological examination. Furthermore, investigations of HARDY and co-workers (1) have proven that we must add to the number of clinical cases an unknown number of infections without illness.

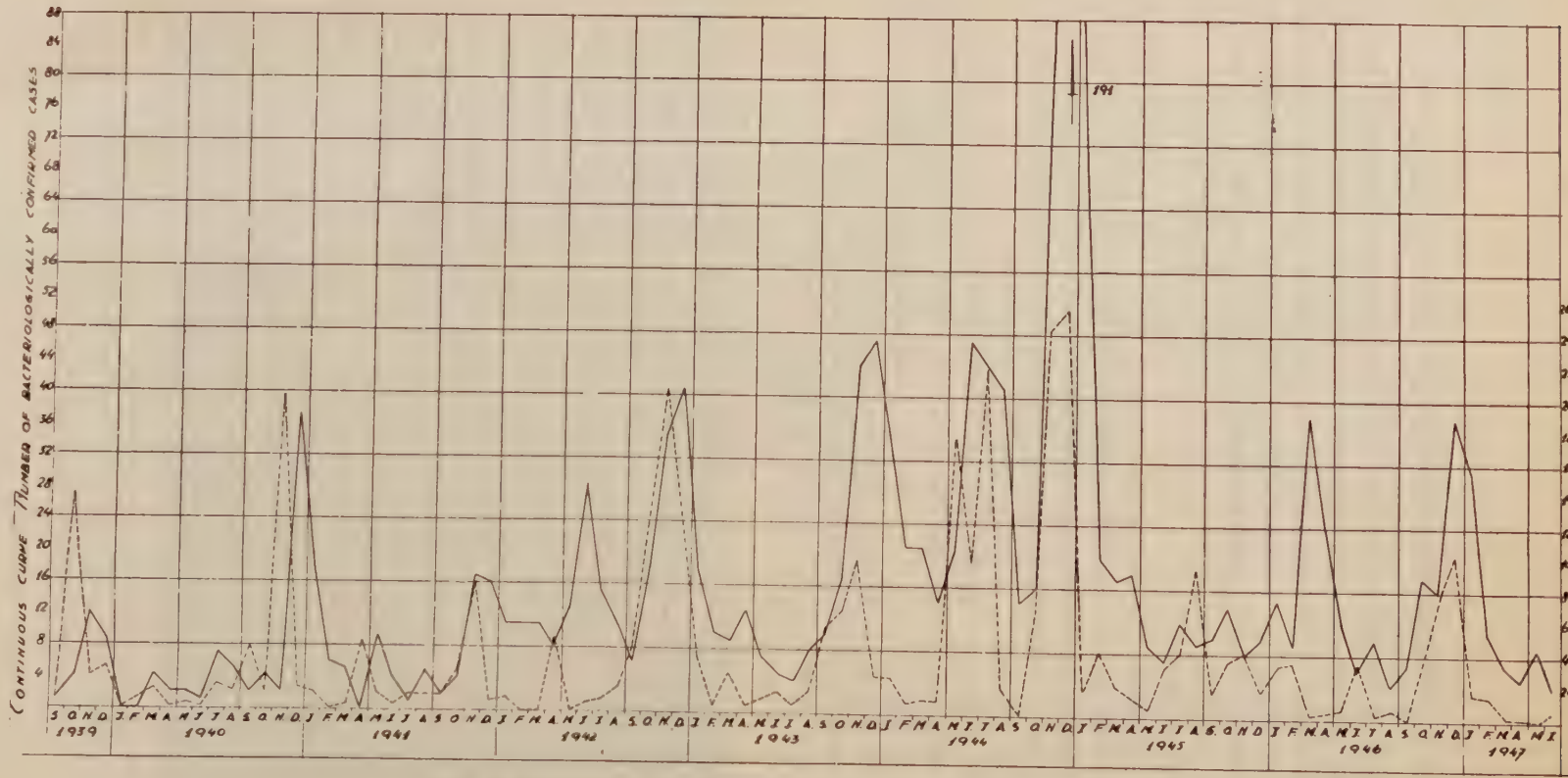


Fig. 3. Shigellosis and Rainfall. Curaçao Sept. 1939—Juli 1947.

Although a great part of the *Shigella* infections are being missed, the number of recognized cases during a certain period may be considered a rough scale for the total number of infections occurring during that time.

With a few exceptions the recorded cases lived in Willemstad and its suburban residential quarters or in the settlements of the „Curaçaose Petroleum Industrie Maatschappij" [1]) i.e. in that part of the island, which surrounds Annabaai and Schottegat.

The question whether „carriers" should be included in the graph has been amply considered. These so-called carriers were discovered at examinations of foodhandlers, hospital personnel and members of patients' families. It seemed, however, probable that the majority of these „carriers" represented fresh infections without illness. Chronic and subacute clinical cases were omitted.

For a correct interpretation of the graph it is necessary to make allowance for the fact that in October 1941 Endo's agar was replaced by Bacto-SS agar as the culture medium of choice (Pot (5)) and that in February 1942 a Newcastle-serum was added to the series already in use. Both factors have caused a marked improvement of results. Therefore an equal number of bacteriologically confirmed cases has a different meaning before and after October 1941.

According to the opinion of many local doctors rainfall is followed by an increase in the number of patients with abdominal disorders. Therefore in the graphic representation the ordinate is at the same time the rainfall in millimeters observed at Fort Nassau. Although important differences in downfall may be recorded at various observation points, Fort Nassau at the entrance of the Schottegat was chosen for its central position between Willemstad and the Oilrefinery.

With due allowance for the inevitable inaccuracy of the figures available it does not seem imprudent to conclude that on Curaçao epidemic outbreaks of Shigellosis may be expected after rainfall.

The next question is how the connection between the two events can be explained. After a period of rain the number of flies rapidly increases in the island. It may perhaps be superfluous to stress here the rôle of flies as mechanical carriers of infection. Shigellae have been isolated several times from flies caught in the neighbourhood of dysentery patients (Smits (7); Taylor (8); Hardy, Watt,

[1]) In Fig. 4 indicated with C.P.I.M.

PETERSON and SCHLOSSER (1)). Some information about the disposal of fecal matter on Curaçao makes it clear that flies have ample opportunity to contaminate themselves with human excreta. The houses of the settlements of the Oilrefinery and part of the houses of Willemstad are connected with a sewerage system. The better houses out of the reach of these systems have cesspools. In a considerable number of houses and huts no attempt is made at sanitary disposal of excreta, which are sometimes collected in kerosine tins and disposed of in the sea and sometimes scattered on the surface of the ground. A certain part of the population considers a clump of bushes on the roadside or on the coast as providing sufficient privacy for defecation.

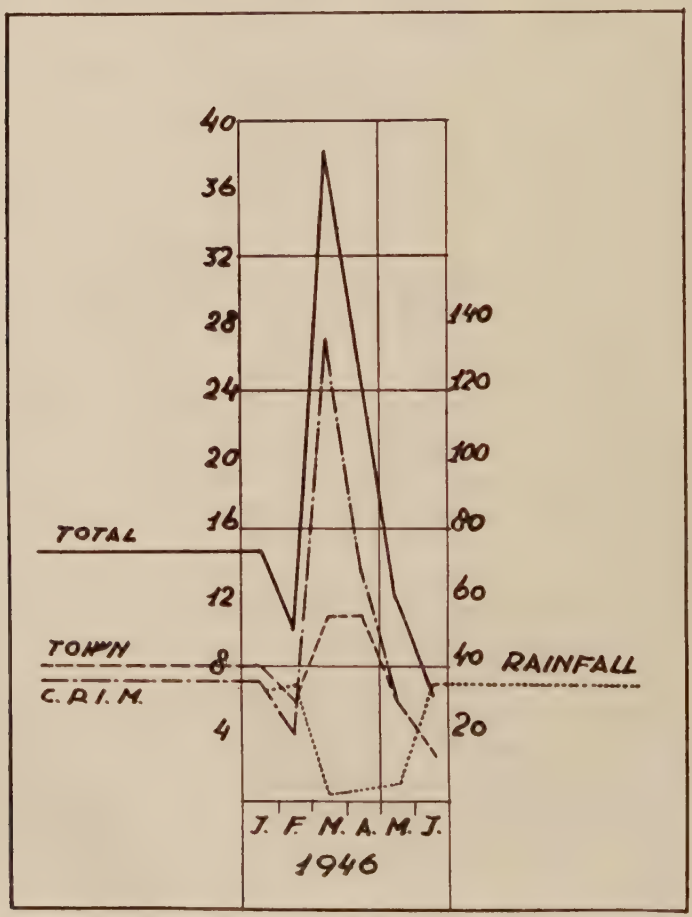


Fig. 4. Shigellosis and Rainfall. Curaçao Jan.—June 1946.

With regard to the survival of Shigellae, the hot and humid environment after rainfall is more favourable than dry weather with abundant irradiation by sunlight. According to KAHN (3) eight to ten days of drying will kill them, while they may survive for several months in a moist environment. Direct irradiation by sunlight is lethal within about ½ hour.

It is clear, however, from the graph that not every rise in the number of infections can be connected with rainfall, e.g. that in March and April 1946. (For details see Fig. 4).

This small epidemic was limited to a residential quarter of the Oilrefinery and caused by *Sh. sonnei*. Examination of the food-handlers revealed that not less than three waiters at the social club harboured *Sh. sonnei* and one out of them *Sh. paradysenteriae*.

Therefore it may be safely concluded that in this outbreak „food, fingers and fomites" played a far more important rôle than „flies". The rôle of flies can be exaggerated. Since their attention has been drawn to these insects some physicians seem to think that flies play the same rôle in bacillary dysentery as mosquitoes do in malaria!

Finally I would correct another misunderstanding concerning dysentery on Curaçao. I have never asserted that amebic dysentery does not occur in this island. The number of cases of clinical amebiasis, however, is small in comparison to that of Shigellosis. Only in exceptional circumstances amebae can give rise to epidemic outbreaks of dysentery. Amebic dysentery is a typical endemic disease.

Summary.

In the island of Curaçao Shigellosis is the most common notifiable contagious disease.

The causative agents are *Sh. paradysenteriae*, *Sh. sonnei* and *Sh. ambigua*.

After rainfall epidemic outbreaks may be expected.

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QUELQUES REMARQUES SUR L'ÉPIDÉMIOLOGIE DE LA POLIOMYÉLITE ANTÉRIEURE AIGUË

par

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(Reçu le 20 juin 1948).

Aux Pays Bas, comme dans plusieurs autres pays, la poliomyélite a augmenté depuis le commencement du vingtième siècle et surtout pendant les derniers vingt ans. Dans l'histoire nous cherchons en vain une telle période de haute morbidité de poliomyélite et rien n'indique une diminution prochaine, qui est même peu probable, parce qu'on observe que cette maladie augmente précisément dans les pays où l'état sanitaire est le meilleur.

Dans notre pays nous pouvons observer qu'en général une épidémie de poliomyélite se présente dans un territoire assez limité; que la durée varie de quatre à six mois et que, pendant ce temps là, la maladie n'a pas de tendance à se propager dans les environs. Un territoire atteint peut avoir une population dense ou une population rare. Un contact régulier entre ses habitants n'est pas nécessaire pour que l'épidémie soit entretenue; il arrive même parfois qu'un grand fleuve coule à travers un territoire atteint.

D'après des expériences sur des singes, au moyen d'injections intrapéritoneales ou intracérébrales de matière suspect, il est bien connu qu'avant l'apparition des prodrômes et pendant quelques jours après celle-ci, le virus se trouve dans les voies aériennes supérieures d'où le malade le disperse en parlant ou en toussant. Pendant la période qui suit, une excrétion en masse a lieu par les selles. C'est pourquoi un malade ou un porteur de germes qui aura un contact avec son entourage, le contaminera, soit en parlant ou en toussant, soit par une main sale, ou indirectement au moyen de mouches.

Plusieurs auteurs pensent que par cette voie la maladie se propage et qu'une petite minorité des gens infectés tomberaient malade de

poliomyélite; ainsi le nombre des gens susceptibles de manifester la maladie serait petit. Si cette théorie expliquait la dispersion de la poliomyélite, il serait encore incompréhensible pourquoi la maladie a augmenté si fortement pendant les dernières dizaines d'années, car il n'y a pas de raison d'admettre que le contact entre les hommes est maintenant plus intime qu'autrefois et tandis que le nombre des mouches a réduit considérablement en particulier dans les villes. En plus de ces arguments, il y a quelques faits qui parlent en faveur de la thèse que le virus, excrété par les malades et par les porteurs de germes, n'est pas capable de produire la forme classique de la maladie directement par voie naturelle.

Au cas ou la dispersion du virus par une personne qui parle ou qui tousse avant l'apparition des prodrômes, serait dangereuse pour l'entourage, il resterait incompréhensible qu'à peu près jamais plus d'un cas de poliomyélite ne se produise dans une classe d'école d'élèves externes. En outre il est extrèmement rare qu'un malade hospitalisé produise de nouveaux cas de poliomyélite parmi les infirmières ou les autres malades, bien qu'il excrète le virus par les selles. La poliomyélite se présente pas non plus dans une famille où un malade rentre de l'hôpital chez-lui, ce qui souvent aura lieu avant que les selles sont négatives.

On peut tenter d'expliquer de pareils faits en supposant qu'après une infection ce n'est que très rarement qu'une poliomyélite manifeste se déclare. Cependant, dans d'autres circonstances, l'infection a des conséquences plus sérieuses. Lorsque des gens vivent ensemble en relations analogues à celles de la vie domestique (famille, internat, caserne, camp de vacances), quelquefois pendant peu de jours, plusieurs membres du groupe tombent malade de la poliomyélite. Il semble que les malades ont été infectés simultanément, tandis que la période d'incubation diffère l'un de l'autre. Dans de telles circonstances l'idée s'impose souvent que des denrées alimentaires ou des boissons ont été la source de l'infection. Apparement, sous de telles conditions, une infection se fait par un virus dont la virulence est très différente du virus excrété directement par les malades et les porteurs de germes. Ces observations suggèrent l'idée que la virulence du virus de la poliomyélite dans des conditions naturelles n'est pas constante, mais qu'il y a des stades de virulence haute et de virulence basse.

Les résultats d'expériences indiquent que l'homme n'héberge pas le virus de manière durable. Probablement le virus doit passer une

certaine phase de sa vie dans le système nerveux de l'homme, ou peut-être dans le système nerveux d'autres vertébrés. Par nature, le virus qui a causé un cas de poliomyélite manifeste a une haute virulence, tandis que le virus qui quitte l'organisme affecté, a une virulence basse. Il en résulte que le virus excrété doit subir une transformation en dehors du corps humain, pour acquérir le pouvoir de produire la poliomyélite chez l'homme par oie naturelle. Nos connaissences sur la vie du virus en dehors du corps humain sont très bornées, il est bien connu qu'il peut vivre ainsi longtemps, même dans des circonstances défavorables.

La grande masse du virus quitte l'organisme affecté par les selles. Autrefois, les selles humaines étaient employées comme engrais; et leur traject était court de l'homme jusqu'à la terre cultivable, où elles étaient enfouies à la bêche. Par contre, pendant les derniers quarante ans, après l'invention du water closet, dans les centres des populations de pays civilisés, on s'est mis, de plus en plus, à employer le système d'égouts aussi pour l'écoulement des matières fécales. Ainsi, par le système d'égouts, le virus peut arriver à la fin aux rivières, aux fleuves et aux lacs et il peut se répandre sur une surface plus étendue qu'autrefois. Il n'est pas étonnant que ce changement se soit accompagné d'une morbidité élevé de la poliomyélite.

D'ailleurs il n'est pas vraisemblable que le virus qui se trouve dans les fleuves et les lacs soit déjà arrivé à la stade de haute virulence, parce que les familles de bateliers fluvials, parmi lesquelles à cause de leur contact fréquent de l'eau contaminée, la fièvre typhoïde n'est pas rare, ne forment pas un groupe qui est particulièrement atteint de poliomyélite. Aux Pays Bas il y a 15 malades habitant un bateau fluvial parmi 340 cas de fièvre typhoïde et parmi plus de 7000 cas de poliomyélite.

Nous ignorons de quelle manière le virus qui se trouve dans les eaux et dans la vase est transporté de là vers la population d'un territoire qui sera plus tard atteint d'une épidémie de poliomyélite. Peut-être un hôte effectue cette tâche et le virus trouve dans cet organisme les conditions favorables à l'augmentation de sa virulence. Si cet hôte excréterait le virus dans notre nourriture, la réalisation de l'infection fatale serait claire.

Selon cette théorie, l'emploi du système d'égouts pour l'écoulement des matières fécales, après l'invention du water closet, est la cause de l'augmentation de la poliomyélite. Avant d'attirer l'atten-

tion des experts techniques à ce sujet, il nous faut connaître mieux la vie du virus en dehors du corps humain. L'étude de ce problème, je la considère comme essentielle à présent et il me semble que l'on y arrivera peut-être lorsque le neurologue, le virologue et l'épidémiologiste collaborent avec le biologiste et le médecin vétérinaire.

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ON THE VARIABILITY OF THE TYPES OF *C. DIPHTHERIAE*

by

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(Received July 8, 1948).

I. Introduction.

In 1931 three types of *C. diphtheriae* were distinguished by Mc. Leod and co-workers (1) differing from each other by colony-appearance on the medium of Mc. Leod and in biochemical behaviour.

Since then many articles have been published about the value of the different properties for typing, the stability of these properties, the type stability and connected with this their usefulness in epidemiological investigation.

Of importance for typing are (*cf.* Anderson *et al.* (1), Frobisher (5)): the colony-appearance on the medium of Mc. Leod, the fermentation of starch and glycogen, the haemolysis of human erythrocytes, the growth in broth, the acidity of broth four days after inoculation, and, according to some, the shape of the cells. Generally the colony-appearance and the fermentation of starch and glycogen are considered to be the best criteria. The different methods used in the various investigations prevent a comparison of the published results.

In examining the colony-appearance most investigators made use of the medium of Mc. Leod; in England those of Downie and Neill are also recommended. Leete (9), for example, using the plates of Neill subdivided the gravis and mitis types. But the investigators, who used the medium of Mc.Leod, came to different conclusions, this partly being due to a too heavy seeding of the plates. When the colonies are too crowded the plates give no typical picture. Carter (3) reported in 1946 the isolation of 14 strains, which had a colony-appearance characteristic of the intermediate

type but with other properties typical of gravis. On the blood and tellurite containing medium after 48 hours daughter-colonies were observed, which when disseminated again showed the colony-appearance characteristic of gravis. This CARTER considers to be a variation of the intermediate to the gravis type. The growth of the colonies may have been restrained, because as can be seen on the photographs the space between the colonies was too small. SIEMENS (17) demonstrated, that super-isolation is indispensable to satisfactory examination of the colonies.

In investigating the fermentation of starch and glycogen some people add the polysaccharides to broth, others take peptone- or serum-peptone-solutions, because bacteria that do not ferment starch or glycogen may still produce small amounts of acid in broth. SCHLIRF (16) advises the use of soluble starch, MUELLER and MILLER (12) on the other hand lay stress on the fact, that good results are only obtainable when making use of insoluble starch.

For the determination of the haemolysis the method of ANDERSON and FROBISHER is generally followed, in which a 2 % suspension of washed human erythrocytes is mixed with the same amount of a two days old broth culture. The mixture is put in a waterbath of 37° C. for one hour and kept afterwards in the refrigerator till next day, when the haemolysis can be read. The results published by HEWITT (8) differ from those of FROBISHER (6). HEWITT used rabbit's blood because the kind of blood would not influence the results. From one of the tables, however, it can be seen, that one out of three gravis strains weakly haemolysed the rabbit's erythrocytes; the human erythrocytes were not haemolysed by any of the three strains. To obtain comparable results a uniform method has to be applied.

About the value of colony-appearance, fermentation of starch and glycogen and haemolysis opinions differ considerably. MAY CHRISTISON (10) thinks, that the property of starch fermentation is a more reliable criterion for typing than colony-appearance. MENTON and co-workers (11) even doubt the type-specifity of the colony-appearance on account of the fact, that they observed no correlation between the colony-appearance and the biochemical behaviour. GUNDEL and LIEBETRUTH (7) on the contrary consider the investigation of the colony-appearance as sufficient for typing and leave the biochemical properties out of consideration.

In a very thorough investigation FROBISHER (6) did not succeed

in finding other more reliable biochemical means of typing.

EWING (4), MURRAY (13), ROBINSON and PEENEY (14) and others studied the antigenic structure. They showed, that the types can be serologically distinguished from one another, but that each type is divided into serologically different groups. According to ROBINSON and PEENEY the antigenic structure is the most constant property, which does not change notwithstanding morphological or biochemical variations.

The occurrence of atypical strains gave rise to the question to what extent the types must be considered constant. ANDERSON, FROBISHER, MURRAY and SIEMENS saw no variation between one type and another. SIEMENS considers the types of *C. diphtheriae* as sub-species and not as fixates.

Others report type variation; it partly concerns typical strains which, kept for some time or sub-cultured under unfavourable circumstances, obtained morphological and biochemical properties characteristic of bacteria belonging to one of the other types. BINGEL (2) observed type variation during some minor epidemics in children's homes and concluded that the types change *in vivo* namely that the gravis bacteria turn via intermediate into mitis bacteria, which in the end become diphtheroid bacteria. These variations would be caused by increase of the patient's immunity. The results of the epidemiological research of 1940 by Professor RUYS (15) clearly prove, that carriers of diphtheria bacteria of different types may infect one another and in this way it may appear, as if type variation has occurred. Similar observations in patients are therefore by no means convincing.

II. OWN INVESTIGATIONS.

During a period of six months in 1946 and four months in 1947 all strains isolated in routine work at the laboratories of the municipal public health department at Amsterdam were typed.

Media for isolation and typing.

The media of LÖFFLER and CLAUBERG were used for isolation, that of MC.LEOD for typing. All strains were examined upon the fermentation of starch.

The media of DOWNIE and NEILL, which are frequently used in England and should both be suitable for typing at isolation, were compared with the medium of MC.LEOD.

The plates of DOWNIE gave the following results: of the 49 isolated strains 13 were not isolated from the DOWNIE medium and 5 not from that of CLAUBERG. Although from so small a number no conclusions can be drawn, the medium of CLAUBERG seems more suitable for isolation.

Half of the strains isolated on the medium of DOWNIE had colonies, the appearance of which was not in accordance with that on the medium of MC.LEOD, while the colony-appearance on the latter was in accord with the biochemical properties. In view of the great difference no further attempt was made to type the bacteria upon their isolation and only strains in pure culture were inoculated on both media. The unsatisfying results might perhaps be explained by the fact, that the colonies were hampered in their normal development by the colonies of other bacteria in the vicinity. Only 3 of the 97 pure strains thus disseminated had different colony-appearance on both plates, in which cases the biochemical behaviour was in accordance with the colony shape on the medium of MC.LEOD.

During the weeks, in which the medium of NEILL was used for simultaneous typing and isolation 146 strains were isolated. Thirty of these strains had grown only on the medium of CLAUBERG, two strains only developed on the NEILL plates. In more than 15 % the colony-appearance on the medium of NEILL was different from that on the medium of MC.LEOD. Besides, the characteristics of the colonies could be more clearly distinguished on the plates of MC.LEOD. With these observations in mind the results of the typing done by LEETE with the aid of the medium of NEILL can be more readily understood.

This investigator would probably not have made a subdivision of the gravis type into six and of the mitis type into two subgroups, if he had used the medium of MC.LEOD. For typing the latter is the most suitable.

Fermentation of starch and glycogen.

The method recommended by SCHLIRF was followed. In a 1 % solution of peptone, which contains 0.3 % NaCl and 0.2 % Na_2HPO_4 1 % starch or glycogen is dissolved. Each culture tube contains 5 ml of the solution sterilised at 100° C. for half an hour. After the culture has grown for a certain time the acidity is titrated with a 1/40 n solution of NaOH, the indicator being phenolphthalein. The quantity of NaOH necessary for neutralisation is used as a

measure for the fermentation. Within each series control tubes must be present, in which no bacteria have been grown and which are to be titrated simultaneously. The method used here only differed from that of SCHLIRF in that not only after 5 days but also after 12 days of incubation the acidity was determined.

Three strains of each type were tested. Two mitis strains and three intermediate strains proved to produce small amounts of acid in the starch-containing liquid, which was prepared with readily soluble starch; glycogen was not fermented. The use of soluble starch was recommended by SCHLIRF. This starch is partly split and may contain traces of glucose. The titrations were repeated with soluble and insoluble starch at the same time. No acid was produced by the mitis and intermediate bacteria in the liquid containing insoluble starch. 42 gravis, 49 mitis, and 20 intermediate strains were tested to determine the quantity of NaOH, necessary for the neutralisation of the acid formed by each of the three types.

With the mitis and intermediate strains the quantity of NaOH was never more than 0.5 ml above the blank value. With several gravis strains the acid produced after incubation for 5 days scarcely exceeded the blank value, while 12 days after inoculation free acid could be demonstrated. So it is useful to repeat the titrations after some days. The interval of 7 days has been arbitrarily chosen, perhaps a shorter incubation may be sufficient.

Fermentation of glucose.

No difference was observed between the fermentation of glucose by the intermediate strains and by the gravis and mitis strains.

Haemolysis.

None of the gravis and intermediate bacteria, of which the haemolitic properties were examined, haemolysed human erythrocytes. Of 20 strains of the mitis type four did not and one did partly haemolyse the erythrocytes. In the other 15 cases haemolysis was complete. On account of these results and the results obtained in investigating the strains, which could not readily be grouped, it seemed justified to set some value to a positive haemolysis for typing.

Atypical strains.

During 6 months in 1946 669 strains were typed, of which 7 %

atypical ones and in 1947 during 4 months 313 strains with 9 % atypical ones. If the colony-appearance was atypical or not in accordance with the biochemical properties, the fermentation of starch and glycogen by means of titration, the colony-appearance in superisolation, the growth in broth and the haemolysis were investigated. Owing to lack of test animals we could not test the virulence of the atypical strains isolated in 1947.

The strains which could not be typed at first examination, can be divided into five groups:

1) Strains with gravis colony-appearance, which did not ferment starch and glycogen. These might be considered as bacteria of the gravis type which lost this fermentative property. It is, however, possible that some strains originate from another type, and have obtained a changed colony shape; these therefore would not belong in the group of gravis bacteria. By considering these strains as atypical erroneous typing may be lessened.

2) Strains with colonies of the mitis type colouring starch solution with litmus pink. From the results of the titrations these bacteria proved to produce small amounts of acid in the solutions of soluble starch, but not to ferment insoluble amylum. Of the 24 strains 3 had a somewhat rough growth, which made it necessary to group them as atypical. The other 21 strains could be considered as mitis bacteria.

3) Strains with their morphological characteristics only evident at superisolation. Of some strains the colonies had a mitis appearance with rough surface and finely crenated edge, at superisolation either a typical mitis or a typical gravis appearance. In some cases the smooth colonies changed at superisolation to gravis colonies or to the atypical colonies just mentioned. The latter ones agree with the strain Amsterdam XXXI, isolated by SIEMENS, and so actually belong to the 4th group.

4) Strains with atypical colony-appearance. Of 14 strains the colony-appearance differed from that of the mitis type by a somewhat rough surface and slightly irregular edge. Two strains had colonies whose appearance resembled those of the intermediate type but differed from them by their large size and black colour.

5) Lastly it must be mentioned, that in two cases a mixture of two types was found. In one throatswab gravis and mitis bacteria were demonstrated; another one seemed to contain the same mixture. However, after subculturing a mitis colony the colonies proved to

have an atypical appearance at superisolation and after keeping them for 6 months the colonies were characteristic of the gravis type. Starch was only slightly fermented, glycogen not at all. After subculturing a colony with gravis appearance no change was noted; the biochemical properties of this strain were of the gravis type. Thus the mixture consisted of bacteria of the gravis type and atypical bacteria, which are to be considered as variants of the gravis type.

III. Variability of typical strains.

By means of some experiments the possibility of changing one type into another was investigated. In case of variation it was intended to determine to what extent the variants could be considered as stable.

Influence of keeping in broth and subculturing only once a week.

The experiments described by Pesch were repeated. Pesch observed variation of the intermediate to the mitis type when culturing in broth and subculturing every 8th day. No such change was observed by us.

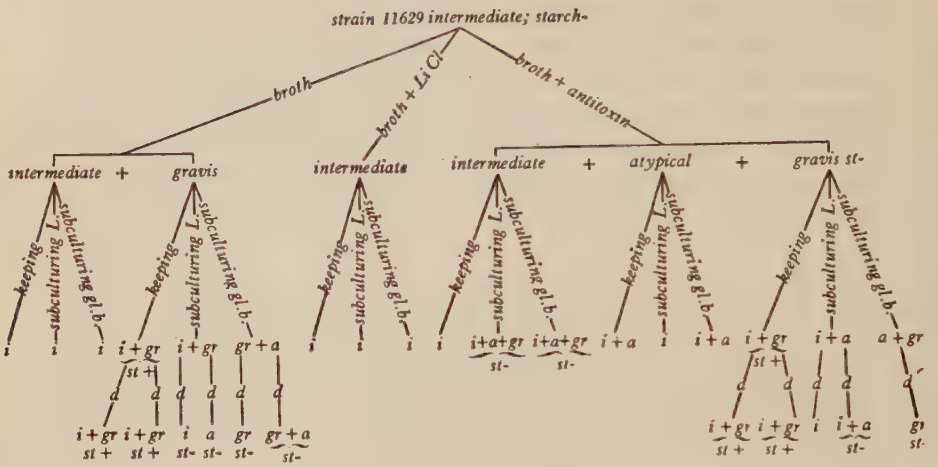
Influence of culturing in liquids containing starch or glycogen.

Culturing in peptone solutions containing 1 % starch or glycogen did not influence the fermenting properties of mitis and intermediate strains.

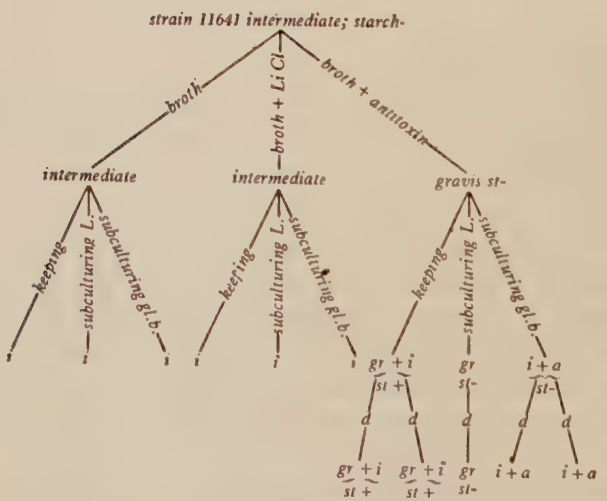
Influence of LiCl and antitoxin.

Two strains of the mitis type, two of the gravis and two of the intermediate types were inoculated in broth, in broth containing 1 % LiCl and in broth containing 100 A.U. antitoxine per ml. The cultures were kept in the incubator for eight days and then they were subcultured. After this procedure had been followed for seven weeks the morphological and biochemical properties were examined. The strains of the mitis and gravis type did not show any change. The properties of the intermediate strains were the following.

The culture of strain 11629 in broth containing LiCl had not changed. The bacteria cultured in broth had colonies with gravis



Variation scheme I. Strain 11629. i = intermediate; gr = gravis; a = atypical; st = insoluble starch; L = Löffler serum; gl.b. = glucose-broth; d = disseminated.



Variation scheme II. Strain 11641. i = intermediate; gr = gravis; a = atypical; st = insoluble starch; L = Löffler serum; gl.b. = glucose-broth; d = disseminated.

appearance and colonies of the intermediate type. The antitoxin containing broth culture had a mixture of colonies of gravis, intermediate and atypical appearance. The atypical colonies had a rougher surface and a more strongly crenated edge than the original ones. The biochemical properties had not changed. The same results were obtained when the experiment was repeated.

After subculturing every other day on LÖFFLER-serum and in glucose broth for six weeks a partial return to the original colony-appearance was observed. Change of the biochemical properties was not seen here either.

Some of the colonies with gravis appearance were subcultured and kept in a stab-culture in serumagar. After 4 months those strains had acquired the property to ferment starch and fermented glycogen to some degree, while the colonies were of the gravis and intermediate type. When subcultured the colonies with gravis appearance and those with intermediate appearance showed a mixture of both colony types. The strains had maintained their virulence.

The other intermediate strain (11641) behaved in much the same way. The difference was, that the broth culture had not changed and the antitoxin containing broth culture had colonies of gravis appearance only. After being kept in stab-culture for four months the variant had colonies of both types and fermented starch.

Seven strains of the mitis, seven of the gravis and six of the intermediate type were subcultured in antitoxin-containing broth according to the above mentioned procedure. Of those only one gravis strain showed a change, the colonies had a mitis appearance, differing from the mitis type by a rough surface. The biochemical properties had not changed.

So most of the typical strains cultured in unfavourable circumstances did not vary. Only with the two intermediate strains No. 11629 and 11641 the morphological changes were attended by biochemical variation.

This does not prove variation of one type into another; such variation could only be proved by demonstrating antigenic changes in the same sense. Lack of test animals prohibited this investigation. Besides, it may be possible that both strains were wrongly typed as intermediate strains, but really were atypical gravis strains.

IV. Variability of atypical strains.

The 77 atypical strains isolated in 1946 were kept in serumagar in stab-culture; the time of observation varied from 6 to 12 months. The colonies of two strains showed changes. In one case the colonies, which at isolation had gravis appearance, had acquired an atypical appearance. The other change occurred in one of the strains isolated from a mixture which has already been described and of which the atypical colonies changed to the appearance characteristic of gravis. The properties of the other bacteria remained the same.

The experiments described while discussing the variability of the typical strains were also performed with 7 atypical strains. In one case the gravis appearance of the colonies changed to a rough mitis appearance by culturing in LiCl-containing broth. A mixture of the two colony shapes was present after frequent and rapid subcultures. 2 atypical strains were both isolated from one patient with an interval of a few weeks. They had large black colonies of the intermediate type and biochemical properties characteristic of the gravis type. After culturing in broth and in broth with antitoxin both showed colonies of gravis appearance. This appearance was maintained throughout subculturing during 4 months. This change must be considered as a return to the original colony appearance.

The results of the experiments show that in most cases the bacteria of the gravis, mitis and intermediate type as well as the atypical ones have a great stability in vitro.

Under special circumstances a change of the colony-appearance of a few strains could be obtained, which change was in two cases accompanied by variation of the biochemical properties.

Conclusion.

From this investigation it is evident that most strains of *C. diphtheriae* can be easily typed; with the typing of some strains difficulties have been encountered. By close examination of the properties some of these can be typed after some delay, superisolation being indispensable.

The use of insoluble starch is essential in the fermentation test. All bacteria which differ in one or more respects from the typical ones must be labelled as atypical. This also concerns gravis strains which do not ferment starch and glycogen. If the bacteria are thus

typed erroneous typing can be minimized, though faults will be inevitable.

The typical as well as the atypical bacteria have great stability. For the moment we wish to consider the types as fixates rather than as subspecies. Notwithstanding the observation of changes *in vitro* in some cases, the types appear to be sufficiently stable for epidemiological investigation.

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ISOLATION OF A STRAIN OF INFLUENZA-A-VIRUS FROM THE TRACHEA IN A CASE OF INFLUENZA PNEUMONIA IN THE WINTER OF 1947 [1])

by

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(Received September 13, 1948).

Introduction.

After an illness of seventy-two hours' duration a man (K), aged 27, died on February 4, 1947, in the Clinic of Internal Medicine of the University Hospital at Leyden, the cause of death being tracheo-bronchitis, with broncho-pneumonia in both lungs due to *Staphylococcus aureus*. Particulars about the case history and the pathology of this case have been given elsewhere (Mulder and Verdonk (11)). As this rapid death of a young man, consequent upon infection of the respiratory tract and the lungs with staphylococci made us think of influenza, part of the trachea together with portions of all the lobes of both lungs were post mortem examined for the presence of influenza virus.

Isolation of a strain of influenza virus from the mucosa of the trachea in ferrets.

The material was stored for 16 hours at —130° C. The mucosa of the trachea was ground in half saline, half broth with 100 units of penicillin per ml and was next inoculated intranasally in a ferret. The remainder was stored at —130° C. Amniotic inoculations in the

[1]) With finantial support of the Institute for Preventive Medicine, the State Department of Science, the Jan Dekker fund, the Curaçao fund for Preventive Medicine and the Department of Medical Research Philips van Houten, Weesp.

chick embryo could owing to circumstances not be performed until seven months afterwards. Rigid isolation of the animals was possible.

After three days the ferret got fever and severe rhinitis, and a strain of influenza virus was isolated in passage ferrets and seven months later from the turbinate of this first ferret (stored at —130° C.) in the amnion of 13 days old chick embryos.

From the third ferret-passage we obtained the strain of the influenza virus by direct inoculation in the allantoic sac of the chick embryo. The strain was regularly passed in the allantoic sac, and after a number of passages it infected eggs in a dilution of 10^{-8} to 10^{-9}. A second ferret, infected in February 1947 with the original emulsion of trachea mucosa, also became ill, and a fortnight after the infection the serum contained immune bodies against the isolated strain (hemagglutination-inhibition test).

In September 1947 we did not succeed in isolating the virus immediately in the amnion from the original emulsion of the trachea mucosa (two attempts with respectively 3 and 4 amniotic passages in 13 days old chick embryos).

Ferret-inoculations with ground lung tissue.

Not until we had the mouse-passage strain at our disposal (see below), were we in a position to obtain a clear idea of what had happened in the inoculations of lung tissue in ferrets.

The first ferret, inoculated with it on February 5, 1947, did not become ill, but after five days it showed a high fever peak (104° F.) without rhinitis. It was then not possible to pass the turbinates of this animal on other ferrets. The post-infectious serum of this ferret had a fairly high titer (96) against the mouse-passage strain (which practically did not show non-specific inhibition by ferret sera), and it also showed a reasonable titer (64) in the mouse protection test with the homologous mouse-passage strain (calculated from the mortality within ten days, four mice being used per dilution).

After six months the lung emulsion (stored at —130° C.) was once more passed to ferrets, but we did not succeed in isolating a virus strain. Passage ferrets (5 passages) did not show any immune bodies, and inoculations from the turbinates into the amnion of 13 days old chick embryos remained negative. Nor was it possible after seven months to isolate a virus strain from the lung emulsion into the amnion (3 passages). Hence there is reason to assume that

the ,,lung virus'' at —130° C. had died in the original material, as opposed to what we know about A-strains. Possibly this is also true for the virus in the mucosa of the trachea. It is known that some B-strains in original garglings may also die off comparatively rapidly after being thawed from low temperatures (HIRST (6)). FRANCIS e.a. (5) also failed to isolate A-strains in the chick embryo in March 1947, though they did easily succeed in the ferret.

ADAPTATION OF THE STRAIN IN THE MOUSE LUNG.

The influenza-A-strain, isolated in ferrets at Groningen (Netherlands) in 1939 (MULDER (9)), and three A-strains of 1941 (VAN BRUGGEN e.a. (1)), could easily be adapted to mice. This was not the case with the 1947-strain. Table I gives a survey of these attempts.

TABLE I.

Mouse adaptation of the strain K (1947 Nl).

Inoculum	Number of mouse-passages necessary for obtaining lethal pneumonia	Control-inoculations
3rd ferret-passage	Failed after 7 passages	7th mouse-passage in ferret: negative
6th ferret-passage	Failed after 14 passages	10th-mouse-passage in ferret: negative 14th mouse-passage in amnion: negative
3rd ferret- and 4th allantoic-passage	7	5th mouse-passage in allantois: positive
3rd ferret-and 24th[1]) allantoic-passage	7	All mouse-passages in allantois: positive
3rd ferret-and 29th allantoic-passage	Mice of the first passage die. Failed after 7 passages	Allantoic inoculation of 5th, 6th and 7th mouse-passages: negative

The fact was made use of that the presence or the absence of the strain to be adapted can easily and quickly be demonstrated in the course of the lung passages by inoculation in chick embryo. Out of

[1]) See: J. WINSSER, Antonie van Leeuwenhoek 14, 214, 1948.

five passage experiments it was only possible on two occasions to adapt the strain to the mouse lung (4th and 24th allantoic-passage).

Here we observed the phenomenon, already described by DUDGEON e.a. (3) in adapting strains of influenza B, that in the first passage of the egg virus the mice may die with the macroscopic aspect typical for influenza pneumonia, while the following passages live on, without showing lung consolidations at the post mortem. This phenomenon is probably due to the fact that in the first inoculation with infected allantoic fluid the quantity of the virus is very great (see table I, last experiment). The microscopic aspect of the mouse lungs of the successful adaptations was characteristic of influenza (criteria of STRAUB (15, 16)). After 13 passages the mouse strain killed mice in a dilution of 10^{-2} (ten per cent lung emulsion), after 23 passages in a dilution of 10^{-3}.

ANTIGENIC COMPOSITION OF THE STRAIN K (1947 NL).

The strain is indicated with K (1947 Nl) giving the first letter of the patient's name, and indicating the year and country of isolation.

PREPARATION OF THE ANTISERA.

The antisera were prepared by inoculating ferrets under narcosis intranasally with 1 ml infected allantoic fluid (0.5 ml in either nostril), and bleeding them after a fortnight. Most titers were high, except of the strain GATENBY (1937 England).

THE HEMAGGLUTINATION-INHIBITION TEST AFTER HIRST.

For the sake of rapidity of working and economizing material a micro method in tiles of porcelain was followed (MULDER and GOSLINGS (10)), which proved most satisfactory in the course of the investigation. The statistical error of this method yielded as standard deviation for the virus titrations about 10 per cent, the standard deviation for serum immune body titration being about 14 per cent (VAN DER VEEN). Comparison with the HIRST-SALK method (titration in tubes) gave values which agreed very well with the serum titrations. An advantage of the micro method is also that pattern reading is extremely clear (from + to ++++), so that for example the determining of the number of agglutination units of the virus always turns out constant. The rapidity and the economical use of materials of this method renders it possible, with few persons, to carry out a large number of experiments in one

day, always checking during the immune body titration how many agglutination units of the virus were actually used [1]).

As we found that the titers of the immune sera were lower when the virus was immediately mixed in the serum fowl cell dilutions, the immune serum and the virus were mixed before adding the red cells. The mixture remained for half an hour in the ice box (+ 4° C.). The drawback of this method was that the titer of the non-specific hemagglutination inhibition in the serum also rose considerably. When afterwards we learned how to neutralize this non-specific inhibition (see below), the method had only advantages. In all the serum tests about 3 agglutination units of virus were used.

TABLE II.

Cross hemagglutination-inhibition tests with pairs of ferret-sera and strains of influenza-virus (4 experiments) *).

Experiment	Pairs of sera (Prae- and post-infection)	Strains		
		K (1947 Nl) (ferret-egg)	PR 8(Infl.A)	Lee (Infl.B)
1	Ferret anti K (1947 Nl)	256/1024	8/64	16/16
2	Ferret anti PR 8 (A)	768/1536	24/6144	48/96
3	Lee (B)	768/768	. . .	48/1536
4	Patient Berg **)	128/8	2048/64	16/8

*) The sera in these experiments had not been freed from non-specific inhibitor. The titer of the sera is expressed as the reciprocal of the highest dilution which with 3-agglutination units of the virus give no agglutination-pattern.

**) The serum Berg (exp. 4) originated from a patient suffering from influenza-(staphylococcal) pneumonia and was again taken three months later. Hence the diminishing in titer of this second serum.

At first the antigenic classification of the strain caused great difficulties in the hemagglutination technique, as the egg-passage strain (from the third ferret-passage) was non-specifically inhibited by most normal ferret sera in dilutions from 384 to 1536. Continued allantoic-passages did not eliminate this. Consequently even a classification as A or B virus was not possible with this technique, as

[1]) For this advice we are indebted to Dr H. Wolff, Institute of Tropical Medicine, Leyden.

also the antiserum of the strain K (1947 Nl) appeared to have a very low titer against PR 8 (1934 U.S.) (table II). The strain proved to belong to the A-group, as was shown by the complement fixation test with ferret sera against the strains PR 8 (influenza A) and LEE (influenza B), and also by the hemagglutination-inhibition test with a patient's serum of another case of influenza A (table II, experiment 4, and table III). The mouse-adapted strain, however, was not or hardly inhibited by the non-specific serum inhibitor. The highest ,,titer'' of 12 normal ferret sera against this strain amounted to 12 on two occasions only. The work was mostly continued with the mouse strain that was obtained from the third ferret and the 24th allantoic-passage, which was afterwards passed again in the allantoic sac of chick embryo.

Table III.

Cross-complementfixation tests with pairs of ferret-sera and strains of influenza-virus (one experiment) [1].

Pairs of ferret-sera (Prae- and post-infection)	Strains		
	K (1947 Nl) (Ferret-egg)	PR 8(Infl.A)	LEE (Infl.B)
Ferret anti K (1947 Nl)	<24/384	<24/96	<24/<24
Ferret anti PR 8	<24/384	<24/6744	<24/<24
Ferret anti LEE	<24/<24	<24/<24	<24/384

[1]) The titers are expressed as the reciprocal of the final serum-dilutions in the tests.

NEUTRALIZATION OF THE NON-SPECIFIC INHIBITOR IN IMMUNE SERA.

An accurate antigenic analysis of the isolated strain did not seem possible to us until the non-specific inhibitor in ferret sera could be neutralized. We were aided by BURNET's discovery (2) that a filtrate of *Vibrio cholerae* contains enzymes which are capable of destroying the aspecific inhibitors in sera. This discovery was developed into a simple routine method, in which the non-specific inhibition is made to disappear by the addition of a filtrate of *Vibrio cholerae* (strain OGAWA, kindly sent to us by Dr W. AEG. TIMMERMAN) to a ferret serum, the immune titers being preserved (MULDER and VAN DER VEEN (12)). The study of the neutralization of non-specific inhibitors by means of potassium periodate (HIRST (7)) was taken up a short time ago.

Technique of the neutralization of non-specific inhibitors in sera (see also Burnet (2)).

Sixteen hours' old cultures of the strain of *Vibrio cholerae* are scraped from the agar slants. The agar is assembled in a gauze bag and the fluid pressed out through a Seitz disc. The titer of the filtrate is determined by mixing twofold serial dilutions with a red cell solution (0.5 per cent), and then adding a suitable dilution of influenza virus.

A sufficiently active filtrate is mixed (in a proportion of from 2 : 1 to 5 : 1) with immune serum, and checked with heated Lee virus. For about 18 hours this mixture is kept at 37° C., and then heated for an hour at 56° C., so as to destroy the enzymatic action of the filtrate.

Antigenic comparison of the ferret-egg-strain and the ferret-egg-mouse-passage strain K (1947 nl).

In the cross tests between the ferret-egg-strain and the mouse-passage strain we were confronted by the remarkable fact that both the anti sera against the ferret-egg-strain and those against the mouse-passage strain showed a considerably lower titer against the former than against the latter. Table IV shows the cross tests between the two strains.

TABLE IV.

Cross-hemagglutination-inhibition tests with the ferret-egg-strain and the adapted mouse-strain (1 experiment) [1].

Pairs of ferret-sera (Prae- and post-infection)	Strains	
	K (1947 Nl) (Ferret-egg)	K (1947 Nl) (Mouse)
K (1947 Nl) (Ferret-egg)	<12/320	<12/1790
K (1947 Nl) (Mouse)	<12/240	<12/3590

[1]) The titer of the sera is expressed as the reciprocal of the dilution which with 3-agglutination units of the virus give a two-plus agglutination pattern (maximal agglutination-pattern noted as four-plus). All sera freed from non-specific inhibitor.

The serum against the adapted mouse strain had been made from the 13th mouse-passage and the 9th allantoic-passage. We obtained

practically the same results with various sera against different ferret-egg-strains and mouse-passage strains. It is not clear why the ferret-egg-strain should show such low titers, but it must be a quality of the strain itself, seeing its antiserum has a good titer against the derivative mouse-passage strain. A distinct difference in antigenic composition, however, is out of the question.

CROSS HEMAGGLUTINATION-INHIBITION TESTS WITH OTHER A-STRAINS.

Table V gives a survey of these tests [1]). From it can be seen that the strain K (1947 Nl) has an antigenic composition deviating so considerably from those found before the year 1947, that it must be considered possible that the strain is a separate representative in the A-group. In order to confirm this, we require more extensive

TABLE V.

Cross-hemagglutination-inhibition-tests with the strain K (1947 Nl) and pre-1947-A-strains *).

Pairs of ferret-sera (Prae- and post-infection)	Strains						
	K (1947 Nl) (Ferret-egg-mouse)	WS (1933)	Talmey (1937)	Christie (1937)	Gatenby (1937)	PR 8 (1934)	Shope (Swine)
K (1947 Nl) (Ferret-egg-mouse)	<12/5380	<12/40	<12/<12	<12/140	<12/60	<12/60	<12/<12
WS	<12/30	<12/3460					
Talmey	<12/<12		<12/8530				
Christie	<12/<12			<12/3410			
Gatenby	<12/<12				<12/770		
PR 8	<12/<12					<12/13700	
Shope	<12/<12						<12/3070

*) The titer of the sera is expressed as the reciprocal of the dilution which with 3-agglutination units of the virus give a two-plus agglutination pattern (maximal agglutination-pattern noted as four-plus). All sera freed from non-specific inhibitor.

antigenic analysis, in which the pre-1947 A-strains too must be crossed mutually, and the Dutch strain must be compared with 1947 strains isolated in other countries. It will also have to be ascertained whether the antigenic composition of influenza strains is comparable, when having been isolated in different ways (via ferret-mouse or

[1]) We are indebted to Dr C. H. ANDREWES and Dr TH. FRANCIS who kindly sent us most of the pre-1947 A-strains.

via chick embryo (HIRST (6)), and when their ,,passage formulae" (ferret-mouse-egg) are different. A further communication on this subject will appear. Information from other countries, however, (FRANCIS e.a. (5), KALTER e.a. (8), STUART HARRIS and MILLER (17), DUDGEON e.a. (4), RASMUSSEN (13), SIEGEL e.a. (14), LÖFSTRÖM [1]) indicates that in 1947 antigenically different A-strains were found everywhere, although their isolation was effected both in ferrets and in eggs.

Summary.

A description is given of the isolation of an influenza virus strain from the trachea of a patient who died from tracheo-bronchitis with broncho-pneumonia due to *Staphylococcus aureus* (February 1947). On serological analysis the strain proved to be an A-strain with an antigenic composition differing considerably from pre-1947 A-strains.

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[1]) Personal communication.

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A STUDY OF CORYNEBACTERIA FROM HUMAN SOURCES, AND ESPECIALLY OF THEIR SENSITIVITY TO ANTIBIOTICS

by

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Introduction.

It is a well-known fact that human throat, nose and ears are frequently inhabited by bacteria belonging to the genus *Corynebacterium*. A study of corynebacteria isolated from such locations, as well as a perusal of the literature pertaining to the subject (3, 4, 5, 26, 38, 55, 71), shows clearly that this group of microorganisms comprises quite a large number of different types, most of them insufficiently studied and described, among which it is often very hard to distinguish clear-cut species. The first part of the present paper reports a number of personal observations emphasizing this fact.

In a few instances, the use of antibiotics has been of great help to differentiate sharply two or more closely related species. It was thus hoped that a knowledge of the sensitivity of corynebacteria to a variety of specific antibacterial agents might provide useful information about their taxonomic interrelationships. The results of our investigations in this field are reported in the second part of the present paper. They should also be useful in connection with the therapeutics of diphtheria or the treatment of carriers by antibiotics.

Part I.

Diversity of Corynebacteria inhabiting human upper respiratory tract.

1. Characterization of *C. diphtheriae*.

Owing to its pathological significance and to its foremost practical importance, *C. diphtheriae* is, by far, the best known species of the group. It is rather well defined by its typical morphology, as well as by its constant biochemical characteristics. The fundamental work of Barratt (4) and of Okell and Baxter (37) was foremost to establish firmly that virulent or avirulent strains of this bacterial species can be differentiated from all other corynebacteria by their constant specific fermentation reactions, *i.e.*: production of acid but no gas from: dextrose, galactose, maltose and dextrin, but not from: sucrose, lactose or mannitol. Their findings have been repeatedly confirmed ever since (18, 22, 70).

The distinction, within the species *C. diphtheriae*, of three different „types", *gravis*, *mitis* (2) and *intermedius* (29), differing morphologically, biochemically and in pathogenicity (31), completes but does not in the least invalidate the former observations.

The numerous non-pathogenic corynebacteria found in human upper respiratory tract, often grouped under the name „diphtheroïds", are, on the whole, much less well known. Most of the work done about them is of a very practical nature, aiming solely at distinguishing them from the pathogenic *C. diphtheriae*. Numerous procedures have been proposed to reach that aim with a lesser or greater degree of certainty; they have been critically reviewed elsewhere (65), with the conclusion, mainly based on personal research, that the most reliable isolated character permitting the practical differentiation of *C. diphtheriae* from the diphtheroïds, is the morphology of bacterial colonies grown on heated blood-tellurite-agar (69, 72).

It must be borne in mind, however, that identification of *C. diphtheriae* by colony-appearance is feasible only when the culture-medium is wholly adequate (76) and provided one strictly observes all the seemingly trivial details of its mode of preparation (77).

In our own experience, based upon the daily examination, during a period of ten years (50, 51, 53, 68, 73), of swab-cultures inoculated onto our own G.C.T.-chocolate-agar (74), and upon the isolation and

study of several hundreds of strains, any colony displaying the morphological characteristics of *C. diphtheriae*, either *gravis*, *mitis* or *intermedius*, always yielded subcultures possessing the specific fermentative abilities.

2. Characterization of diphtheroïd groups.

On the same culture medium, all the microorganisms of the diphtheroïd group, isolated from human respiratory tract, developed colonies belonging to one out of only five types, each one being sharply different from the other four, as well as from the three types of *C. diphtheriae*. These five diphtheroïd colony types are indefinitely stable upon continued cultivation; they were called type H, V, X, Y and Z (75). Their complete description with photographs are published elsewhere (71). Colonies of any one of these five types never yielded subcultures having fermentation reactions similar to those of *C. diphtheriae*. In most cases, sugar fermentation by our diphtheroïds enabled us to allot them without trouble to any one of the eleven fermentation-groups earlier described by Barratt (4); in a very few cases only we were compelled to set up new fermentation-groups for strains that could not be thus classified. The fermentation properties of each strain were found to be as stable upon continued cultivation as was their morphological type when grown on G.C.T.-agar.

Table I illustrates the complexity of the diphtheroïd group in showing a number of our strains classified by taking into account simultaneously the morphology of their colonies and their ability to ferment a series of carbohydrates. It can thus be seen that many fermentation-groups contain strains belonging to several different morphological types, and *vice-versa*.

It is therefore very hard to define what should be considered as a „species" among those diphtheroïds. Colony morphology sets apart five clearly-cut, stable types, which must, at least, rank as species. On the other hand, fermentation reactions are considered as a character of sufficient importance and stability to define the species *C. diphtheriae* and set it apart from all other corynebacteria. If the value of such a character is regarded as „specific" in a particular case, it should logically take the same importance in every other case within the genus. Unfortunately, the taking into account as „specific" characters both morphological type and fermentative ability would result into a considerable multiplication of the

TABLE I.

Relationships between colony-type and fermenting ability of diphtheroïds.

Serial Number	Fermentation groups [1])							Number of strains giving colonies of type					Total
	Dextrose	Galactose	Maltose	Sucrose	Lactose	Dextrin	Mannitol	H	V	X	Y	Z	
12	+	0	0	0	0	0	0	0	0	1	0	0	1
4	+	0	±	0	0	0	0	0	0	0	0	0	0
3	+	0	+	0	0	0	0	0	1	3	0	0	4
5	+	+	+	0	0	0	0	19	2	2	0	0	23
5a	+	+	±	0	0	0	0	6	0	0	0	0	6
2	+	0	±	±	0	0	0	0	0	0	0	0	0
7	+	0	+	+	0	0	0	4	0	4	1	0	9
7a	+	0	+	+	0	+	0	0	0	1	1	0	2
1	+	+	±	+	0	0	0	12	0	1	2	0	15
8	+	+	+	+	0	0	0	15	7	1	0	7	30
8a	+	+	+	+	0	+	0	1	0	0	0	0	1
10	+	+	±	+	0	0	+	1	0	0	0	0	1
6	+	+	+	+	+	0	0	0	2	0	0	1	3
9	+	+	+	+	+	+	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	157	0	0	2	0	159
Total								215	12	13	6	8	254

[1]) + = acid production; 0 = no acid produced; ± = transient acidity.

number of species, a consequence which is probably far from desirable.

The situation becomes still less clear if additional properties are brought into the picture. Then, the strains, gathered into little groups defined by gross morphology and fermentative ability, are further distributed into smaller units, according to their microscopic morphology and to their ability to produce pigment, grow anaerobically, form ammonia from urea (table II), reduce nitrates to nitrites (table III), form hydrogen sulfide from cystine (table IV), *etc.*

Some of these properties may be of very secondary importance, but some have been considered as „specific" and were thought suitable for differentiating the species *C. diphtheriae* from the whole group of diphtheroïds. Thus, urea-fermentation is never obtained

TABLE II.

Relationships between colony-type and a few morphological and biochemical properties of diphtheroïds.

Properties studied			Number of strains of each type					Total
			H	V	X	Y	Z	
growth on nutrient agar	good	smooth and glistening	59	0	0	0	0	59
		rough and dry — raised	0	8	0	0	0	8
		rough and dry — flat	0	0	0	0	2	2
	poor		24	0	10	2	0	36
microscopy on G.C.T.		short rods	30	0	5	1	0	36
		short rods, granular	3	1	2	0	0	6
		medium rods	40	0	1	0	0	41
		medium rods, granular	3	7	2	1	2	15
		long rods	4	0	0	0	0	4
		long rods, granular	3	0	0	0	0	3
growth in deep agar-cultures		strictly aerobic	71	5	10	2	2	90
		microaerophilic	12	3	0	0	0	15
ammonia from urea		+	73	7	0	1	2	83
		—	10	1	10	1	0	22
yellow endo-pigment		produced	0	3	0	0	0	3
		not produced	83	5	10	2	2	102
		Total	83	8	10	2	2	105

with the species *C. diphtheriae* (25, 34, 42), whereas some diphtheroïds produce ammonia from this substance (21, 75). Conversely, all strains of *C. diphtheriae* examined form H_2S from cystine, whereas the diphtheroïds may or may not produce this reaction (54).

It has long been known also that *C. diphtheriae* produces an acidification of sugar-free culture-media, whereas diphtheroïds have no such activity (46). All our diphtheroïds unable to form H_2S from cystine are at the same time unable to acidify a sugar-free culture-medium, while all our *C. diphtheriae* strains are endowed with both abilities (78). This observation is of importance, since it gives an explanation of the specificity of halo formation by *C. diphtheriae* cultivated on blood-tellurite-agar containing cystine and sodium

TABLE III.

Relationships between colony-type, sugar-fermentation and nitrate reduction by diphtheroïds [1]).

Nitrate reduction [2])	Number of strains in fermentation-group									Total	Type
	I	II	III	V	Va	VI	VIII	XI	XII		
+	5			2	6		1	10		24	H
—	0			0	0		0	0		0	
+	1		0	3		3	2		0	9	V
—	0		1	1		0	2		2	6	
+	5	0		1	2				1	9	X
—	0	1		1	1				0	3	
+							3			3	Z
—							5			5	
Total	11	1	1	8	9	3	13	10	3	59	

[1]) 22 strains of *C. diphtheriae* (10 *gravis*, 10 *mitis*, 2 *intermedius*) gave a positive nitrite-test.

[2]) Nitrite-test: suitably diluted 5-day old cultures in Hiss' serum-water containing 0.5 % KNO_3 are treated with α-naphtylamine and sulfanilic acid.

TABLE IV.

Relationships between colony-type and H_2S-production by diphtheroïds.

Colony-type	Number of strains		
	H_2S +	H_2S —	Total
H (non fermenting)	0	30	30
H (fermenting)	11	4	15
V	4	11	15
X	0	11	11
Z	1	8	9
Total	16	64	80

thiosulfate. Halo-formation under specified cultural conditions was discovered by TINSDALE (56), who pointed out its practical value for the identification of the Klebs-Loeffler's organism; a fact which was completly confirmed by us (52, 77). In opposition to TINSDALE's opinion, however, we found that the mechanism of halo-formation

was different from the agency responsible for the black colour of colonies grown on tellurite medium. This blackening is due to an accumulation of metallic Te, resulting from the reduction of K_2TeO_3, while the halo is made of TeS_2, resulting from the reaction, in acid medium, between K_2TeO_3 and H_2S formed from cystine (78).

3. Comparison of our strains with Bergey's species.

It thus appears that if characters, which are taken as „specific" to define the species *C. diphtheriae*, have the same value to define species among diphtheroïds, the number of these should be considerably increased. At all events, it is evident that the number of diphtheroïd species from human sources is much higher than is assumed by Bergey's Manual (5), in the last edition of which only 5 such species are retained. Many of our diphtheroïds, completely different from the described organisms, are absolutely unidentifiable with Bergey's key.

First, we should point out that three of Bergey's species are not represented at all in our collection of strains isolated from human respiratory tract. All our strains grow freely under normal oxygen tension, although some of them can grow in the depth of shake-agar tubes; therefore, we can eliminate the anaerobic *C. acnes*, which, furthermore, has not been reported as living in the respiratory tract. The relative resistance of this species to the action of penicillin (8) is also in opposition with the sensitivity of our organisms to this antibiotic (see 2d part). None of our strains showed the characteristic pink growth of *C. hoagii* on inspissated serum. None of our strains is indole-producer, none of them is highly pleomorphic as is the rule with *C. enzymicum*.

We are thus left with only two species, namely: *C. pseudodiphtheriticum* and *C. xerose*. Undoubtedly, a large number of our strains, showing colony-type „H" and falling into the non-fermenting group 11, can be identified as *C. pseudodiphtheriticum*. Some of them, however, are atypical, either in giving a poor growth on plain nutrient agar or in failing to produce ammonia from urea. A few of them, finally, must belong to another species, since their cells are long rods, generally club-shaped, with or without polar granules. This last group is homogeneous, giving good growth on plain nutrient agar and fermenting urea. H-type diphtheroïds which ferment sugars are certainly distinct from *C. pseudodiphtheriticum*; whether they should form a single species or be dif-

ferentiated into several ones is difficult to decide now. In fact, they form a group fairly homogeneous (38 strains studied); the cells are short or medium rods (one exception), rarely granular; they usually ferment urea (4 exceptions); but their action on sugars is quite variable.

Our type Z corresponds closely to *C. xerose*; the cells are long rods with polar granulations; urea fermentation is observed with all strains and sugar-fermentation is quite typical (dextrose +; galactose +; maltose +; sucrose +), but for the fact that some strains ferment lactose while some do not.

Our types V, X and Y are entirely different from any of the species described in BERGEY's Manual. Type V is a rather homogeneous group; the cells are short or medium rods, with a granular appearance; growth is good on plain nutrient agar, and in most strains ammonia is produced from urea; fermentation of sugars, on the other hand, is quite variable. A few strains of this group form a bright yellow endo-pigment. They represent the only pigmented strains of our collection. Whether strains of type V should form a single species or be divided into two species on the basis of pigment production is a debatable point.

Strains of type X also form a rather homogeneous group; their cells are short or medium rods, sometimes granular; they grow poorly on plain nutrient agar and do not ferment urea; again, their action on carbohydrates is quite variable. Nevertheless, we are of the feeling that they probably belong to a single species.

Strains of type Y are less frequent; their cells are short or medium solid rods; urea as well as sugar fermentation is variable. They probably belong to a single species also.

In conclusion, our study of diphtheroïds isolated from human respiratory tract shows the existence of some new species in addition to the five ones already described in BERGEY's Manual. Our strains, in fact, can be grouped and named as proposed in table V.

In this grouping, we have brought together, under a single specific name, a number of strains having same colony-morphology but different fermentation reactions. We thought that wide differences of sensitivity to antibiotics within some groups, especially if they would be parallel to differences in fermentative ability, could justify the division of the group into two or more species. Therefore, a study of the action of antibiotics on a few selected strains was undertaken. A study of the sensitivity to antibiotics of several

TABLE V.

A summarized description of species within the group of diphtheroïds from human respiratory tract.

Morphology on tellurite-blood-agar (G.C.T.)		Fermentation of		H_2S production	Growth on plain nutrient agar	Identification
Colonies	Cells	urea	sugars			
H	short, solid rods; with transverse colourless zone.	usually +	none fermented.	—	usually good	*C.pseudodiphtheriticum.*
H	long rods, sometimes granular.	+	none fermented.	usually +	good	*C.elongatum* nov.sp.
H	short or medium rods, usually solid; no transverse colourless zone.	usually +	variable, but some are always fermented.	usually +	usually good	*C.fermentans* nov.sp.
Z	medium or long rods; polar granules.	+	dextrose, galactose, maltose, sucrose, and sometimes lactose are fermented.	most usually +	good	*C.xerose*
V	short or medium rods, granular.	usually +	variable	usually —	good	*C.asperum* nov.sp. (with „*flavum*" var.)
X	short or medium rods, sometimes granular.	—	variable.	—	poor	*C. acuminatum* nov.sp.
Y	short or medium rods, solid.	variable	variable.	?	poor	*C.minutissimum* nov.sp.

representatives of the well defined species *C. diphtheriae* was made simultaneously, in order to obtain information on the range of activity of each antibiotic upon different strains within a single species of the genus, and even upon different strains within each of the „types" constituting the species.

Part II.

Sensitivity of Corynebacteria to Antibiotics.

a) Antibiotics used in this study.

The antibiotics tested in our experiments include: penicillin, streptomycin, tyrothricin, actinomycin and fumigacin. The action of actinomycetin on corynebacteria was reported previously (67). We found that all corynebacteria, heat-killed or alive, could be readily dissolved by actinomycetin under proper conditions; no significant difference of sensitivity was observed between the various strains tested.

The penicillin used was a commercial sample of crystalline penicilline G (Merck); the concentrations tested varied from 1 Oxford unit per ml (*i.e.* 1 u/ml) down to 0.007 u/ml. The streptomycin, also a commercial sample (Bristol), was tested at concentrations ranging from 5 micrograms per ml (*i.e.* 5 γ/ml) down to 0.03 γ/ml. Tyrothricin was obtained as a partially purified product, sold on the Belgian market (Laboratoires Quinta) under the tradename „Septogramine"; concentrations of this rather crude material ranging from 100 γ/ml down to 0.75 γ/ml were tested. Actinomycin (62) was purified and crystallized in the laboratory from aerated submerged cultures of our own producing strains (66, 67); the crystalline preparation was tested at concentrations varying from 5 γ/ml down to 0.06 γ/ml. Fumigacin (60), or helvolic acid (7, 58), was prepared in the laboratory, from stationary cultures of *Aspergillus fumigatus* in Czapek-Dox medium, by adsorption on activated charcoal (Acticarbone) followed by elution with ether first and chloroform next. The active eluate was evaporated to dryness *in vacuo* and the solid residue taken up in ethanol. As this alcoholic solution is likely to contain some gliotoxin along with fumigacin (33, 59), the results recorded have no absolute significance and are expressed merely as dilutions of the stock solution.

b) Strains tested.

A total of 52 corynebacteria were examined; they include 27 representatives of the species *C. diphtheriae* (17 *gravis*, 6 *mitis*, 1 *intermedius* and 3 atypical strains) and 25 diptheroïds. These include 7 representatives of *C. fermentans* (H), 6 of *C. xerose* (Z), 4 each of *C. asperum* (V) and *C. minutissimum* (Y), 3 of *C. acuminatum* (X) and 1 of *C. pseudodiphtheriticum* (H).

c) Technique of the test.

It was thought advisable to use a single method of assay throughout for all strains and for all antibiotics. The sensitivity of each strain to each antibiotic was determined by a serial dilution method, using Hiss' serum-water with dextrose and phenol-red as a culture-medium, as advocated by Fleming and Smith (16). Serial dilutions, in two-fold steps, were made for each antibiotic in 1 ml of the culture-medium. The series of tubes containing the dilutions were thereafter inoculated with one drop each from a 48-hour culture of the strain studied, grown at 37° C. in peptone-water. The test-tubes were then incubated at 37° C. and examined after 18, 24, 48 and 72 hours, both macroscopically and microscopically. The highest dilution of antibiotic inhibiting completely the growth under those conditions was taken as a measure of the sensitivity of the strain under investigation.

d) Experimental results.

The results obtained with each one of our strains are embodied in figure 1 for *C. diphtheriae* and in figure 2 for the diphtheroïds. They will be discussed in the following sections.

e) Action of antibiotics on *C. diphtheriae*.

As far back as 1929, Fleming (15) reported that a strain of *C. diphtheriae* was about 5 times less sensitive to his crude penicillin than *Streptococcus haemolyticus*. With more potent and purer penicillin, Abraham and co-workers (1) observed the same fact; in addition, they reported that a *mitis* strain was about 4 times as sensitive as a *gravis* one.

More recently, rather conflicting reports have been published on the action of penicillin upon *C. diphtheriae*. The growth of six strains, examined by Young and Mood (80), was inhibited by concentrations of the antibiotic varying from 0.002 to 0.03 u/ml. A more extensive investigation, conducted by Buxbaum *et al.* (6),

revealed that the development of 72.2 % from 385 strains tested was checked by concentrations of penicillin between 0.25 and 1 u/ml. Still more resistant were the 10 strains studied by McDaniels (32), since from 2.5 to 640 u/ml penicillin were needed to inhibit their growth.

The same sort of conflicting reports are to be found in the literature about the action of penicillin in experimental (6, 14, 80) or clinical diphtheria (9, 10, 13, 19, 24, 27, 35, 36, 39, 41, 45, 49, 63, 64, 79).

It appears from figure 1 that the 27 strains examined by us are inhibited by penicillin at concentrations ranging from 0.015 to 0.12 u/ml. It is very likely that the discrepancies found in the literature are mostly due to differences in technique. It is rather interesting to note that the range of sensitivity of a group of strains is generally covered by a ten-fold variation of the antibiotic concentration. We can see that the *intermedius* strain is the most sensitive and that the *gravis* group, as a whole, is slightly more resistant than the *mitis* one.

The sensitivity of *C. diphtheriae* to streptomycin has been less studied. According to Heilman (20), *C. diphtheriae* strains are sensitive to concentrations of this antibiotic between 0.375 and 3.75 γ/ml. Robinson (44) found that the growth of two avirulent strains was inhibited by 1.87 and 3.75 γ/ml streptomycin, respectively, whereas a concentration of 15 γ/ml was necessary to check the development of a *gravis* strain. May, Voureka and Fleming(30) observed that the smallest inhibiting quantity of streptomycin for a given strain of *C.diphtheriae* could vary from 1 part in 600,000 (roughly 1.7 γ/ml) down to 1 part in 60,000,000 (roughly 0.017 γ/ml), according to whether the titration was performed in normal broth, or in broth diluted to one tenth with distilled water. The activity of streptomycin on the diphtheria organism was also reported by Richou (43), who advocates the use of this antibiotic for the treatment of diphtheria and the sterilization of carriers. Cases of penicillin and streptomycin resistant diphtheria have been reported, however (63).

All our strains are inhibited by 0.07 to 1.25 γ/ml streptomycin. No difference of sensitivity is demonstrable between the *gravis* and *mitis* groups; the *intermedius* type, in this case, is no more sensitive than the other two.

The sensitivity of *C. diphtheriae* to gramicidin was early reported by Pico (40). According to Schoenbach *et al.* (47), 1 γ/ml tyro-

Fig. 1. The distribution of sensitivity to various antibiotics among different strains of *Corynebacterium diphtheriae*. G: typical *gravis* type; M: typical *mitis* type; i: *intermedius* type; g: atypical *gravis* type (*i.e.*: starch and glycogen not fermented); m: atypical *mitis* type (*i.e.*: starch and glycogen fermented).

thricin inhibits the growth of the Klebs-Loeffler's organism. Jacob (23) states that several strains of *C.diphtheriae* are completely inhibited by 7.5 to 10 γ/ml tyrothricin. On the basis of such *in vitro* findings, tyrothricin was used for the treatment of clinical diphtheria and for the sterilization of carriers (23, 35, 48, 57).

In our experiments, the sensitivity of *C. diphtheriae* to tyrothricin appears rather less than previously reported, since growth of our strains is inhibited by 3 to 50 γ/ml tyrothricin. This may be due in part to the lesser degree of purity of the tyrothricin used, and in part to the fact that our tests were performed in Hiss' serum-water. One knows that serum depresses considerably the activity of one component of tyrothricin, namely tyrocidin, and slightly that of the other one, namely gramicidin (12). The *intermedius* strain is the most sensitive to tyrothricin; the *gravis* type is slightly more resistant than the *mitis* type.

The sensitivity of *C. diphtheriae* to actinomycin was observed by Welsch (66, 67). In the present experiment, growth of all strains was inhibited by 0.12 to 1 γ/ml actinomycin. The *intermedius* strain, again, appears as the most sensitive, but there is no evident difference of resistance between the *gravis* and *mitis* groups.

The action of fumigacin on *C. diphtheriae* has not been reported yet, to our knowledge. All our strains are sensitive to this antibiotic, the *mitis* type, this time, being slightly more resistant than the other two.

In conclusion: 1) The variation of antibiotic concentration needed to inhibit the growth of all our *C. diphtheriae* strains is not very wide; the smallest range of minimal inhibiting concentrations, a four-fold one, is obtained with fumigacin, and the greatest one, a 16-fold one, with streptomycin and tyrothricin. 2) The distribution-curves of sensitivity among our strains are fairly symmetrical in some cases (penicillin, tyrothricin, actinomycin), but very skew in other cases. With streptomycin, one finds ,,exceptional'' strains trailing along the ,,more sensitive'' side of the curve, while the reverse is true with fumigacin. 3) There is some evidence of a ,,type-specificity'' of antibiotic action: with the exception of streptomycin, all the antibiotics tested are more active against the *intermedius* type than against the two others; the *gravis* type is slightly more resistant to penicillin and tyrothricin than the *mitis* one; the reverse is true for fumigacin; equal sensitivity is observed for the two types with streptomycin and actinomycin.

f. Action of antibiotics on diphtheroïds.

Very little is known as yet of the action of antibiotics on corynebacteria other than *C. diphtheriae*. Lovell and Cotchin (28) state that there are wide differences of sensitivity to penicillin among various strains of *C. ovale*. They found, however, that this antibiotic was useful to repress the experimental infection induced in mice by this organism. On the other hand, penicillin did not give entirely good results for the treatment of bovine infections by *C. renale* (17) or *C. pyogenes* (11). The resistance to penicillin of *C. acnes* was early reported by Craddock (8), who described a selective penicillin-containing culture-medium to facilitate the isolation of this micro-organism.

The action of streptomycin on corynebacteria was reported by Robinson (44), who found that various species from animal sources (*C. pseudotuberculosis, C. renale, C. pyogenes, C. equi, C. ovis*) were sensitive to concentrations of 3.75 to 15 γ/ml of the antibiotic. The minimal inhibiting concentrations for species from human sources were recorded as follows: 3.75 γ/ml for *C. pseudodiphtheriae* and *C. xerose*; 7.5 γ/ml for *C. hofmanni*.

We do not know any work dealing with the action of tyrothricin, actinomycin or fumigacin on diphtheroïds.

An examination of figure 2 immediately shows clearly that the range of sensitivity to antibiotics displayed by the whole group of diphtheroïd species is of the same order of magnitude as the one displayed by strains of the single species *C. diphtheriae*. This indicates, from the start, the homogeneïty of the group and suggests that antibiotics will be of little value to differentiate species within it.

A closer study of our results, however, enables one to make some interesting observations. The single strain of *C. pseudodiphtheriticum* (11h) included in our experiments shows the highest sensitivity to all antibiotics, except to streptomycin and fumigacin.

The three strains of morphological type X (*C. acuminatum*) are divided into two groups. Two strains, belonging to fermentation-group 5 (5x) show an equal sensitivity to fumigacin and tyrothricin; while the third strain, which belongs to fermentation-group 1 (1x), is four times more resistant to the first antibiotic and four times more sensitive to the second one. On the other hand, the sensitivity of the three strains is practically the same for the other antibiotics studied. In the case of *C. acuminatum*, therefore, a difference of

Fig. 2. The distribution of sensitivity to various antibiotics among strains of different species and types of diphtheroïds. The numeral indicates the fermentation-group of each strain and the letter its morphological type.

sensitivity to some antibiotics is parallel to a difference in fermentative ability.

Four strains of morphological type Y (*C. minutissimum*) are also divided into two groups: two of them belonging to fermentation-group 1 (1y) and the other two to fermentation-group 11 (11y). Here, however, the sensitivity of the four strains is nearly the same for each antibiotic tested; especially, the sensitivity of a strain from one group may take a value intermediate between those of two strains of the other group. In the case of *C. acuminatum*, therefore, antibiotics fail to emphasize the division of the species suggested by sugar-fermentation.

The same conclusion is arrived at with strains of morphological type Z (*C.xerose*). They include 5 typical strains (8z) and one lactose-fermenting strain (6z). This last one is generally just a little more resistant to antibiotics than the first five ones, but, with fumigacin, its sensitivity takes an intermediate value between those of different group-8 strains.

Four strains of morphological type V (*C.asperum*) again are divided into two groups, two of them belonging to fermentation group 8 (8v) and two others to fermentation group 6 (6v). The sensitivity of the two strains constituting each group is equal, or nearly so, for all the antibiotics tested. On the contrary, the two groups, generally, show a different degree of sensitivity; thus, strains of group 6 are twice more resistant to penicillin than those of group 8; four times more to streptomycin and tyrothricin; but four times less to fumigacin. In the case of *C. asperum*, therefore, the actien of antibiotics emphasizes the existence of the sub-groups already revealed by the study of fermentative ability.

The seven strains from morphological type H endowed with fermentating power (5h), *i.e.*: *C. fermentans*, form a quite homogeneous group, giving perfectly symmetrical distribution-curves of sensitivity to each antibiotic. It must be pointed out that the seven strains constituting this group have identical fermentation reactions. They show a higher sensitivity to streptomycin, tyrothricin and actinomycin than the other diphtheroïds; but, conversely, they display a higher degree of resistance to penicillin and fumigacin. The lesser or greater degree of sensitivity of this little group is especially evident with tyrothricin and fumigacin, in which cases it is responsible for the irregular shape of the distribution-curve of sensibility among diphtheroïds.

In conclusion: 1) the whole group of diphtheroïds appears very homogeneous from the point of view of sensitivity to antibiotics. The variation of antibiotic concentration necessary to check the growth of all these strains belonging to several distinct species is not significantly greater than the variation recorded to obtain inhibition of all the strains from the single *C. diphtheriae* species. The smallest range of minimal inhibiting concentrations, a four-fold one, is observed with actinomycin, while the greatest one, a 32-fold one, is observed with tyrothricin.

2) Evidence of a „species-specificity" of antibiotic action is especially well demonstrated in the case of *C. fermentans*. Strains of this group show clearly a lesser degree of resistance to some antibiotics, and a greater one to some others, than the rest of the diphtheroïds.

3) In two cases, namely with *C. acuminatum* and *C. asperum*, evidence of „intra-specific" selectivity of antibiotic action was obtained. With these two species actual differences of sensitivity to antibiotics were found between groups of strains endowed with different fermentative abilities. In other cases, however, namely with *C. xerose* and *C. minutissimum*, no such parallelism between sensitivity to antibiotics and sugar-fermentation was observed.

4) The significance of these results for taxonomic purposes can not yet be fully assessed, since the number of strains examined from each group is still obviously too small. They indicate, however, that it may be worth while to continue the study on a larger scale in order to investigate the sensitivity to antibiotics of numerous representatives of each of the present species that are not homogeneous with respect to sugar fermentation.

Summary.

1) Some of the characters that set *C. diphtheriae* apart from other corynebacteria are reviewed and discussed; they include: gross morphology after cultivation on heated-blood-tellurite-agar, urea and sugar fermentation, H_2S production, acidification of sugar-free culture medium, formation of halo on cystine-tellurite-agar.

2) The complexity of the group of microorganisms gathered under the name: „diphtheroïds from human respiratory tract" is emphasized.

3) On the basis of: macroscopic and microscopic morphology

after cultivation upon heated-blood-tellurite-agar, urea and sugar fermentation, H_2S production, the group is divided into at least seven distinct species, five of which are newly described ones.

4) The growth of all strains of *C. diphtheriae* studied is inhibited by the following concentrations of antibiotics: penicillin: 0.015 to 0.12 u/ml; streptomycin: 0.07 to 1.25 γ/ml; tyrothricin: 3 to 50 γ/ml; actinomycin: 0.12 to 1 γ/ml. A crude solution of fumigacin inhibited the development of those strains after dilution between 1 in 800 and 1 in 100.

5) *Intermedius* type is more sensitive to all antibiotics studied, streptomycin excepted, than the two other types.

6) *Gravis* strains are more resistant than *mitis* to penicillin and tyrothricin; conversely, they are more sensitive to fumigacin. *Gravis* and *mitis* types are equally sensitive to streptomycin and actinomycin.

7) The same range of antibiotic concentrations which is active on *C. diphtheriae* is also sufficient to check the growth of all the diphtheroïds tested.

8) Some species (*C. fermentans*) are clearly more sensitive to some antibiotics (streptomycin, tyrothricin, actinomycin), but more resistant to others (penicillin, fumigacin), than the rest of the diphtheroïds.

9) Within a given species, groups of strains endowed with different fermentative abilities may show differences of sensitivity to antibiotics (*C. acuminatum*, *C. asperum*). With other species showing variable fermentation reactions, such parallelism may not be found however (*C. xerose*, *C. minutissimum*).

10) The significance of these observations for taxonomic purposes can not be appreciated fully as yet, but a more extensive investigation along the same lines promises to be fruitful.

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SOME ASPECTS OF THE ADAPTATION OF INFLUENZA VIRUS ONTO MICE [1])

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Shortly after the introduction of the ferret as an animal suited for influenza research (SMITH, ANDREWES and LAIDLAW) successful attempts were made to adapt the influenza virus onto mice (ANDREWES, LAIDLAW and SMITH) after some passages through the ferret.

Especially the experimental influenza research has made a frequent use of this cheap animal, although just this frequency has given rise to difficulties above all when the incubated hen's egg competed with this species as an experimental animal and as culture medium.

For the successful infection of mice, the intranasal instillation of 0.05 ml of virulent material under ether anesthesia is necessary and they will show after 2 days or later, but never earlier, characteristic lung lesions (,,consolidation'') which will prove fatal when the lungs are almost totally affected. The number of preceding ferret passages varies, according to the characteristics of the isolated strains.

HIRST (4) was unable to adapt the strains Ala 41 and Kil 41, both isolated from throatwashings of patients during the American 1941 epidemic, onto mice before the 6th ferret passage.

FRANCIS and MAGILL (3), on the other hand, were able to adapt their New York strains from December 1936 onto mice after only one ferret passage. These strains proved to be fatal for mice as early as the second mouse passage. Within a few days the lungs showed characteristic lesions. Mice were inoculated intranasally

[1]) Communication of the Workteam of Acute Respiratory Diseases of the Institute for Preventive Medicine.

with the original throatwashings from which one of these strains had been isolated by ferret inoculation and blind passages were performed each 4th to 5th day, and in the 4th mouse passage small but typical lung lesions could be detected and in the 6th passage death occurred.

By ferret inoculation MULDER (11) isolated an influenza virus strain from the tracheal mucosa of a young man who died in 1947 of an acute fulminant staphylococcal pneumonia. From this virus (K-strain) the first nasal scrapings we received were of the 8th ferret passage (KF_8), stored at low temperature (liquid air). We performed with KF_8 8 blind passages in mice, reinoculated every 2 days fresh mice with lung suspension of mice from the preceding passage. As none of the mice during 8 passages showed any lung lesions, the experiment was brought to a close, and it was suggested that ,,somewhere'' the virus had been lost.

A ferret, inoculated intranasally with lung suspension of the 4th mouse passage remained healthy; after inoculation of a bacteriologically sterile lung suspension of the 5th mouse passage in the allantoic sac of embryonated eggs the presence of influenza virus could not be demonstrated with the hemagglutination test; the same negative result was obtained with the material of the 6th and the 1st mouse passage, all stored at —20° C.

We then received material from the 5th ferret passage, KF_5, in which we were unable to demonstrate influenza virus.

We had more luck with material of the 3rd ferret passage. From this, egg passages had already been made successfully and with the 23rd egg passage (KF_3E_{23}) we performed 4 blind mouse passages. This experiment was ended by irrelevant reasons and we started with a new egg passage, KF_3E_{24}.

We always inoculated 5 mice intranasally, killed 3 of them after 2 days (the 3 survivors served as controls and if they had not died spontaneously within 10 days, they were sacrificed on the 11th day for the examination as to the presence of characteristic lung lesions) and inoculated lung suspension in fresh new mice and in the allantoic sac of embryonated hen's eggs (table I).

Up till the 10th mouse passage the presence of influenza virus in the mouse lungs was checked by inoculating eggs, always with positive result (hemagglutination). From the 6th mouse passage characteristic lung lesions could be observed (indicated as +, + +, + + +, resp. + + + +).

Table I

KF_3E_{24} → 5 mice. controls: 2; no lung lesions.	3: killed after 2 days. Lung suspension M_1 → eggs. (allantoic fluid contained after 2 days influenza virus: hemagglutination titer not estimated).
Lung suspension M_1 → 5 mice. controls: 2; no lung lesions.	3: killed after 2 days. Lung suspension M_2 → eggs it. positive (titer [1]) 1 : 60—1 : 120).
Lung suspension M_2 → 5 mice. controls: 2; no lung lesions.	3: killed after 2 days. Lung suspension M_3 → eggs it. positive (titer [1]) 1 : 240).
Lung suspension M_3 → 5 mice. controls: 2; no lung lesions.	3: killed after 2 days. Lung suspension M_4 → eggs it. positive (titer [1]) 1 : 480).
Lung suspension M_4 → 5 mice. controls: 2; no lung lesions.	3: killed after 2 days. Lung suspension M_5 → eggs it. positive (titer not estimated). Etc.

[1]) Method Mulder, Goslings and Enserink (6).

Sixth passage: controls +, + + +; 7th passage: controls + + + (died), + + (sacrificed on the 11th day). From the 8th passage all control mice died regularly with almost totally consolidated lungs, at first 8 to 9 days after inoculation and then after gradually shortening intervals, the phase of ,,virus fixe'' being reached in the 13th mouse passage ($KF_3E_{24}M_{13}$), i.e. death came in 3-4 days after inoculation.

Our K-strain belongs, as appeared from the mouse protection test (table II), to the influenza A virus. It is not identical, however, either with the classical W.S. strain or with the $P.R._8$ strain (only the homologous ferret antiserum neutralized the K-strain in high dilution).

This mouse adaptation, although performed with some trouble, does not offer anything particular. It offers, however, an outlook on the nature of adaptation. If the adaptation of a micro-organism to some animal species is wanted, it will often be necessary to perform a number of blind passages, after which the pathological signs and symptoms will show more and more clearly and in continued passages the animals may die. It may be imagined that the micro-organism will multiply very slowly in the first passage, most

Mouse protection test with K virus $K^{P_3}E_{54}M_{12}$.

A with homologous ferret serum

Nr. of mouse	1 : 2	1 : 10	1 : 50	1 : 250	1 : 1250
	number of days, following inoculation	number of days, following inoculation	number of days, following inoculation	number of days, following inoculation	number of days, following inoculation
	1 2 3 4 5 6 7 8 9 10 11	1 2 3 4 5 6 7 8 9 10 11	1 2 3 4 5 6 7 8 9 10 11	1 2 3 4 5 6 7 8 9 10 11	1 2 3 4 5 6 7 8 9 10 11
1	i.c.	nl	nl	i.c.	i.c.
2	nl	nl	nl	**1**	**4**
3	nl	nl	nl	nl	**4**
4	nl	nl	nl	nl	**4**
5	nl	nl	nl	nl	**3**
6	nl				
7	nl				
8	nl				
9	nl				

B. with ferret anti B (LEE) serum

Nr. of mouse	1 : 2	1 : 10	1 : 50	1 : 250	1 : 1250
	number of days, following inoculation	number of days, following inoculation	number of days, following inoculation	number of days, following inoculation	number of days, following inoculation
	1 2 3 4 5 6 7 8 9 10 11	1 2 3 4 5 6 7 8 9 10 11	1 2 3 4 5 6 7 8 9 10 11	1 2 3 4 5 6 7 8 9 10 11	1 2 3 4 5 6 7 8 9 10 11
1	4	1	i.c.	4	4
2	4	3	3	4	4
3	4	4	4	4	4
4	4	4	4	3	4
5	4	4	4	4	4
6	3	**2**	4		4
7	3	**3**	4		3
8					4

C. with ferret anti-Slagter serum

Nr. of mouse	1 : 2	1 : 10	1 : 50	1 : 250	1 : 1250
	number of days, following inoculation	number of days, following inoculation	number of days, following inoculation	number of days, following inoculation	number of days, following inoculation
	1 2 3 4 5 6 7 8 9 10 11	1 2 3 4 5 6 7 8 9 10 11	1 2 3 4 5 6 7 8 9 10 11	1 2 3 4 5 6 7 8 9 10 11	1 2 3 4 5 6 7 8 9 10 11
1	nl	nl	4	3	3
2	nl	nl	4	2	2
3	nl	nl	4	3	3
4	nl	nl	4	4	4
5	nl	nl	4	4	**4**

D With pig-anti W. S. serum

Nr. of mouse	1 : 2	1 : 10	1 : 50	1 : 250	1 : 1250
	number of days, following inoculation	number of days, following inoculation	number of days, following inoculation	number of days, following inoculation	number of days, following inoculation
	1 2 3 4 5 6 7 8 9 10 11	1 2 3 4 5 6 7 8 9 10 11	1 2 3 4 5 6 7 8 9 10 11	1 2 3 4 5 6 7 8 9 10 11	1 2 3 4 5 6 7 8 9 10 11
1	i.c.	nl	4	4	4
2	nl	nl	4	4	4
3	nl	nl	4	4	4
4	nl	nl	3	4	4
5	nl	nl	**2**	4	**2**

E. with ferret anti W. S. serum

Nr. of mouse	1 : 2	1 : 10	1 : 50	1 : 250	1 : 1250
	number of days, following inoculation	number of days, following inoculation	number of days, following inoculation	number of days, following inoculation	number ofdays, following inoculation
	1 2 3 4 5 6 7 8 9 10 11	1 2 3 4 5 6 7 8 9 10 11	1 2 3 4 5 6 7 8 9 10 11	1 2 3 4 5 6 7 8 9 10 11	1 2 3 4 5 6 7 8 9 10 11
1	**1**	**2**	4 nl	4	4
2	nl	nl	nl	4	4
3	nl	nl	nl	4	4
4	nl	nl	nl	4	4
5	nl	nl	nl	4	**2**

F. with ferret anti P.R.$_8$ serum

Nr. of mouse	1 : 2	1 : 10	1 : 50	1 : 250	1 : 1250
	number of days, following inoculation	number of days, following inoculation	number of days, following inoculation	number of days, following inoculation	number of days, following inoculation
	1 2 3 4 5 6 7 8 9 10 11	1 2 3 4 5 6 7 8 9 10 11	1 2 3 4 5 6 7 8 9 10 11	1 2 3 4 5 6 7 8 9 10 11	1 2 3 4 5 6 7 8 9 10 11
1	nl	nl		4	3
2	nl	nl	nl	nl	4
3	nl	nl	nl	**2**	4
4	nl	nl	nl	**3**	3
5	nl	nl	nl	**3**	4

controls

Nr. of mouse	only K virus 1 : 2
	number of days, following inoculation
	1 2 3 4 5 6 7 8 9 10 11
1	4
2	4
3	3
4	4
5	4
6	3

Code: All surviving mice have been sacrified on the 11th day, and the lung lesions are expressed in figures (**1** = + **2** = ++ etc.), or as nl = no lung lesions. The lung lesions of mice, which died spontaneously within 10 days after infection are expressed in figures 1—4 only. i.c. = intercurrent death. Slagter = influenza virus-strain, isolated by Prof. MULDER in Groningen in 1941; this strain belongs to the influenza A group.

of the particles dying off and only a few surviving; the latter are presumably variants possessing a greater faculty for adaptation onto the new host and in further passages a selection goes on, finally the selected variant getting the mastery, inducing symptoms of illness and anatomical lesions in the new host, even causing death of the animal.

Is this theory of value for the influenza virus?

Influenza virus may be adapted, after some ferret or egg passages, in 5-20 mouse passages to the latter animals, in a sense that a virulent suspension of mouse lung, after intranasal inoculation, will be lethal for mice in a dilution of 10^{-6} and will cause lung lesions in a further hundredfold dilution (10^{-8}).

Smorodintseff and Ostrovskaya (8) pointed out that the highest virus titer in the lungs of mice, infected with a lethal dose of influenza virus, is reached 24-48 hours after the instillation of the virus suspension (subinfection experiments in fresh mice), although no lung lesions are present.

Taylor (10) observed that the lungs of mice, inoculated intranasally with a large dosis of influenza virus, will reach the maximum virus titer (mouse lethality titer) 24 hours after inoculation. The amount of virus in the lungs of a mouse, inoculated with a lethal dosis, 24 to 48 hours after infection, was 10^7 M.L.D. (Mouse Lethality Dosis = the smallest amount of influenza virus that will cause death of 50 per cent of the mice [1]) after intranasal inoculation) and this amount was kept constant until death. Instillation of a sublethal dosis resulted in a maximum of only $10^{4.9}$ M.L.D. 48 hours after infection, although the virus had multiplied no less than one million times! Although such a mouse lung contains an amount of influenza virus sufficient to kill 76000 fresh mice, the animal, however, can survive the infection.

The use of the egg as a medium for the growth of influenza virus has thrown a better light on the happenings during the adaptation period in the mouse. At any moment of the adaptation period the amount of virus present in the mouse lung can be estimated by the egg titration (so called 50 % infective dosis), the ,,in ovo'' titer being quite independent from the stage of adaptation of the virus onto mice.

Hirst (4) studied the adaptation of an influenza A strain, Ala 41,

[1]) Reed and Muench (7).

onto mice. With this strain, isolated from throatwashings of a patient, during an influenza epidemic in Alabama in 1941, by 2 amnion passages and 5 subsequent allantoic passages, 6 mice have been inoculated intranasally; these animals were sacrificed after 3 days and with the lung suspension 6 fresh mice have been inoculated, etc., whilst at the same time the infective titer of the lung suspension for eggs was estimated. The egg infective titer of the allantoic fluid (5th egg passage) was $10^{7.5}$. With this fluid the first group of mice has been infected (0.05 ml intranasally). After each mouse passage the fresh suspension of mouse lung was checked for mortality titer in a new group of mice (M.M.T.), for lethality titer in the same group of mice (M.L.T.) and for the egg infective titer (E.I.T.).

HIRST noted that the lung suspension of the first mouse passage by subinoculation in mice caused no death and no lung lesions although the E.I.T. was nearly as high as those of the original allantoic fluid (5th egg passage). In subsequent mouse passages the M.M.T. and M.L.T. rose quickly to reach their maximums in the 6th mouse passage: the M.M.T. was about 10^{-4}–10^{-5}, the M.L.T. was always 2 logarithms higher. The E.I.T. on the other hand remained constant from the first passage, namely 10^{-7} to 10^{-8}. After intranasal inoculation in mice the virus multiplies quickly, independently of its being a first mouse passage or a good adapted virus. Mice inoculated with a sublethal dosis of adapted virus will survive, although their lungs will contain, some days after the instillation, an enormous amount of virus. According to TAYLOR (10) this can be explained by assuming that in a mouse inoculated with influenza virus only a relatively low number of cells of the bronchial epithelium will be infected by the virus. The virus particles multiply quickly but remain locked up in the cells for some days. In this period an immunity develops and when the epithelial cells burst and disintegrate, the greater part of the liberated virus particles will be neutralized by antibodies. This immunity, however, is not stable, there is only an equilibrium with the virus.

One may conclude that the virus titer of the infected mouse lungs is proportional to the number of primarily infected epithelial cells. An inoculation wrongly performed with a lethal dosis of virus may be the reason that the animal will survive.

More difficult to explain is the nature of the adaptation. In HIRST's view the egg- or ferret-adapted virus will be substituted in the mouse by a variant, responsible for the pathogenic characteristic

of the virus for mice, which should multiply quicker than the original egg- or ferret-passage virus which should be completely substituted by the pathogenic variant.

It is difficult to establish whether in a group of influenza virus particles a number of them will possess deviating characteristics and under certain circumstances will determine by their predominance the characteristics of the virus strain as a whole, or whether every virus particle will possess a certain variability, a certain adaptation power, this explaining a.o. ,,virulence'' as a degree of adaptation.

When mice, inoculated with a sublethal dosis of influenza virus are subinoculated after a few days intranasally with an indifferent sterile fluid, a number of them will die. TAYLOR infected mice with 0.06 M.L.D. and instilled 1-10 days afterwards an indifferent fluid (distilled water, NaCl in different concentrations, broth with 10 % horse serum). Now the batches of mice suffered losses and TAYLOR observed: 1st that the mortality was highest (90 per cent) when the instillation of the indifferent fluid was made on the 4th day, not earlier or later; 2nd that death did not occur within 3 to 4 days after the instillation of the indifferent liquids; 3rd that the virus content of the lungs rose considerably.

Aggravation of lung lesions subsequent to instillation of Tyrode solution 1 to 2 days after inoculation of mice with a weak strain of influenza virus had already been observed by STRAUB (9). TAYLOR also observed that the microscopical lung lesions found after instillation of an indifferent fluid, a few days after inoculation of a sublethal dosis of influenza virus, closely resembled those in mice infected with many lethal doses of virus. This phenomenon may probably be explained by the assumption that instillation of an indifferent fluid on the moment that the virus particles after multiplication will be liberated, may promote the spreading of the virus particles to the unaffected epithelial cells, this minimizing or neutralizing the effect of the developing immunity. As to TAYLOR this phenomenon may give an explanation for the pathogenesis of the influenza pneumonia in man: the secondary infection in an influenza bronchitis promotes the liberation and spreading of the virus particles through the bronchi so that many other epithelial cells will be affected by the virus. The virus bronchitis stimulates the invasion of bacteria and the latter promotes the virus bronchitis quickly giving rise to a fatal pneumonia. The existence of a certain degree

of uniformity in the pathological picture offered by the influenza-pneumonia, not depending on the kind of secundary invaders, should advocate this theory.

A practical application of the instillation of indifferent fluids is their use in adaptating a weak influenza virus strain onto mice or other animals.

BURNET and his co-workers studied the adaptation phenomenon in eggs. Using embryonated hen's eggs for the isolation of new strains of influenza virus, it will first be necessary to make amniotic passages. The influenza virus occurs in the human being in the so called ,,O'' (original) phase and will multiply only in the amniotic cavity of the incubated egg. This ,,O'' virus agglutinates guinea pig erythrocytes much more strongly than chicken red cells and is not able to grow in the allantoic sac. After a few (as a rule 2) amniotic passages the ,,O'' phase virus is already substituted by the ,,D'' (derivate) phase virus. The ,,D'' phase virus multiplies easily in the cells of the chorio-allantoic membrane of the chicken embryo, agglutinates chicken red cells better than guinea pig erythrocytes and has a low or no virulence for man. According to BURNET the ,,D'' phase virus merely arises when the influenza virus has the opportunity to multiply sufficiently (10^5 to 10^6 times).

The ,,O'' type virus is incapable to multiply in mice and according to BURNET, this is the reason that a few preceding egg- or ferret-passages are necessary for the adaptation onto mice; in the ferret more and more ,,D'' phase virus will be produced and as a rule after 2 to 5 passages there will be an amount of ,,D'' phase virus adaequate for a successful infection of mice. It is remarkable, however, that HIRST observed exactly the contrary when adaptating the influenza A strain N.Y. 43 onto mice; the agglutination titer (chicken erythrocytes) of the perfused (for the removal of all red cells) mouse lungs remained constant in each passage but the agglutination titer with regard to guinea pig red cells, which was already higher in the initial mouse passages, increased during the further passages. In het 30st mouse passage, in which the influenza virus had a very high virulence for mice, the hemagglutination titer for guinea pig red cells was 32 times higher than those for chicken cells. This virus strain, N.Y. 43, should be in the ,,O'' phase in the mouse, conversely to BURNET's view.

It appears from the mouse adaptation of strain Sinai 45 by HIRST that influenza virus strain may show a varying biological

behaviour. This strain had been isolated from the throatwashings of a patient by amniotic passages. It could be adapted only with difficulty to the chorio-allantoic membrane of the chick embryo, and even after its adaptation onto eggs, HIRST did not succeed in adaptating it onto mice: he succeeded herewith only after 3 ferret passages. After 15 subsequent mouse passages small but characteristic lung lesions appeared, deaths, however, not occurring.

In the mouse the influenza virus undergoes a change of its biological characteristics and the question may be raised whether passages may change its antigenic structure. In almost every influenza epidemic of some importance, virus strains may be isolated which, after serial mouse passages, have a different, but related, antigenic structure and it is of importance whether in one and the same epidemic from an equal number of foci various variants of influenza virus are present or that the virus in its serial passage in man, is changed in its antigenic pattern. HIRST examined a number of egg-isolated influenza A strains of the American epidemic 1940-'41 and concluded that all strains which have been passed only in eggs, possessed an identical antigenic structure (in vitro hemagglutination tests) whereas two strains isolated in the same epidemic by ferret and mouse inoculation, differed mutually as well as from the egg-isolated (and passed) strain. To confirm the presumption that the mouse passage is the source of antigenic differences, HIRST studied the strains Ala 41 and Kil 41, which passed in eggs only, did not show any differences in antigenic structure. Ferrets were inoculated with the original throatwashings stored in dry ice and after 6 ferret passages HIRST succeeded in adapting the strains onto mice. Strain Ala 41 was now passed 30 times through mice, strain Kil 41 12 times. Homologous ferret antisera were prepared and with the agglutination-inhibition test significant differences in antigenic patterns could be detected between both strains, when compared mutually as well as with the original egg-passed strains Ala 41 and Kil 41.

For studying the occurrence of antigenic differences in influenza virus strains the mouse is not a suitable animal and it is desirable to isolate influenza virus by preference in hen's eggs; if the original virus containing material is stored at low temperature, one can, in case of unsuccessful egg passages, as yet inoculate ferrets. When in influenza virus strains, isolated during one and the same epidemic, r e a l antigenic differences are detected, they may be variants or mutants.

The continuous passage of influenza virus in man during an influenza epidemic, may undoubtedly give rise to variants or mutants but these aberrant strains, as a rule, do not get a chance to play an important role in such an epidemic (interference phenomenon) and the chance of isolating such a mutant is very small. The occurrence of a new influenza epidemic is partly dependent on the state of immunity of the population, partly on the presence of mutants against which the immunity is not sufficient. BAETJER (1) isolated during the 1947 epidemic in the U.S. strains, a.o. the FM_1 strain, which possessed a distinctly different antigenic structure, compared with strains from former epidemics, although all strains belonged to the influenza A group. The influenza vaccin used up to this epidemic failed in the 1947 epidemic, the persons vaccinated with this vaccin showing only a low antibody titer against the 1947 strains (mutants, variants).

In the experiment mutants are never observed; however often an influenza strain is passed in mice, the original antigenic structure is always preserved. It may be pointed out from HIRST's experiments, that a mutation only occurs during the transmission of the virus from one animal species to another.

Summary.

The author describes the adaptation onto mice of an influenza A strain, isolated by MULDER from the tracheal mucosa of a man, who died from an acute fulminant staphylococcal-pneumonia and discusses the nature of the adaptation phenomenon.

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(From the Centraal Bureau voor Schimmelcultures, Baarn, Holland).

OBSERVATIONS ON SUBMERGED WATER MOULDS IN THE NETHERLANDS

by

AGATHE L. VAN BEVERWIJK

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Introduction.

Holland with all its water seems an interesting place for the investigation of aquatic fungi. Nevertheless not much was known about the occurrence of water moulds in this country. Oudemans (1904) in his „Catalogue" reports *Achlya prolifera*. Boedijn (1921) records *Saprolegnia monoica*, *Saprolegnia ferax* and *Sapromyces Reinschii*, in 1923 Boedijn adds six more species to this list. Since then no water moulds from Holland have been recorded.

In 1943 and 1944 the following investigations were carried out at the Centraal Bureau voor Schimmelcultures at Baarn, with interruptions caused by the claims of other work at this laboratory.

I. Methods.

Collections were made from various places in Holland, but mainly from two localities: a pond in the botanical gardens at Baarn and a small pond near Ommen in the Eastern part of the Netherlands. The pond in the botanical gardens was chosen because it was near the laboratory and collections could easily be made all through the year. The water of this pond is in connection through a culvert with the dikes of the Eempolder. As a pond in botanical gardens may contain plants from all parts of the world this might not be considered a suitable place to study the water moulds of Holland. For this purpose the second locality is more appropriate: a pond in the garden of the private estate of Eerde near Ommen. This pond was originally a patch of marshy ground cut off from the river Regge before this was canalized. Some twenty years ago it was deepened to form a pond and since then left undisturbed.

The pond is surrounded by birches and shrubs; not many human beings or animals approach it and even water-birds seldom go there [1]). As will be seen from the following reports, the curious thing is that the less common species such as *Achlya oligacantha*, *Saprolegnia asterophora* were all collected from the pond at Eerde.

The hydrogen ion concentration of the water samples was determined in the laboratory by means of a quinhydron electrode. The pH of the water of the pond at Eerde varied from 7.0 to 7.5, that of the pond in the botanical gardens at Baarn from 6.8 to 7.4. The pH of the waters from which the other collections were made was about 7.0, unless otherwise stated. Once an *Achlya colorata* was collected from a fen; the pH of its water was 4.9, but no regular collections were made from ,,acid waters'' (term employed by LUND, 1934). The hydrogen ion concentration of the tap water in which the cultures were kept was about 7.2.

In isolating and culturing these fungi, the methods recommended by COUCH (1927) have been applied with good results. Water samples of 40 ml were poured out into Petri dishes and baited with halves of boiled hemp seeds. After about five days mycelial growth becomes visible on the seeds; sporangia develop and after some weeks sexual organs are formed. Sometimes these rough cultures can be identified as they are; mostly, however, swarmspores of different species land on the same hemp seed. These mixed cultures must be separated. Under a dissecting microscope a single sporangium about to discharge its spores is cut away and transferred to a fresh culture dish. As spores of another species may adhere to the sporangium wall, this process should be repeated several times before one can be certain to have a monofungus culture. Cultures obtained in this way are still contaminated with bacteria. Even after frequent subculturing from agar plates, I never succeeded in obtaining a really bacteria-free culture, pure as it might seem during the first few weeks.

Many methods to free fungus cultures from bacterial contaminants have been described, by BLANK and TIFFNEY (1936), by MELIN (1932), RAPER (1936) and by SCHULTZ (1942). Several of these methods were tried, in an attempt to purify the cultures of the Phycomycetes of the C.B.S. In my experience the only reliable

[1]) I wish to thank PH. BARON VAN PALLANDT VAN EERDE for this information and for the free access he gave me to his garden.

method is to make single spore cultures. A sporangium is allowed to discharge in a drop of sterile water on a flamed slide. Directly after the spores have emerged, the empty sporangium is removed from the drop. With a thin platinum loop a drop of this spore-suspension is gently spread out on a cornmeal agar plate. The next day under a binocular microscope the germinating spores can be cut out and transferred to a fresh cornmeal agar plate (COUCH 1927).

Most Phycomycetes, however, are not greatly hindered in their development by the presence of bacteria.

Blastocladiaceae and *Leptomitaceae* that are attached to their substratum by rhizoids, require another method of collecting. Apples and medlars that have been lying in ditch water for some weeks are often covered with small white tufts. When seen under a binocular microscope these white pustules appear to consist of a rhizoid bearing water mould, overgrown with bacteria and algae. Often it is not possible to identify the fungus in this state. The fruits are then washed in running tap water and put in sterile water with other fresh fruits, crab apples proving very useful. After about a week white tufts develop on the fresh fruits. These new cultures are not quite as contaminated as the original growth and in better condition for the development of reproductive organs. It is of importance to keep these cultures at a low temperature to retard bacterial growth as much as possible. We have not succeeded in obtaining a pure culture of a rhizoid bearing water mould.

II. Some Remarks on Phycomycetes in general and on the Taxonomy of the *Saprolegniaceae*.

Early mycologists classified the Phycomycetes amongst the algae, hence the name which means algal fungi.

The great diversity in the Phycomycetes makes it impossible to generalize as to their morphological characteristics. As the present investigation mainly deals with Oomycetes, some features of this group will be given here.

The mycelium is coenocytic, septa are formed only to delimitate the reproductive organs.

Asexual reproduction occurs by means of sporangiospores, formed in sporangia. In the order of the *Saprolegniales* these spores are motile and provided with two cilia, they may be called zoospores or swarmspores. Another asexual spore form, the chlamydospore or gemma, occurs in various genera of the Phycomycetes.

Sexual reproduction: in the female gametangium (oogonium) one or more oospheres differentiate. From the male gametangium (antheridium), when present, a fertilization tube pushes out, pierces the oogonium wall and discharges its contents into the oospheres. The oospheres mature into thick walled oospores lying free in the oogonium.

The distinction of the genera in the *Saprolegniaceae* is mainly based on the characteristics of the sporangia and the behaviour of the zoospores. The species within each genus are separated by the morphological characters of the sexual organs. Identification of a species is, generally speaking, only possible if one can observe the sporangia as well as the sexual organs of the fungus.

The two main genera *Saprolegnia* and *Achlya* were established by Nees von Esenbeck (1823). In *Saprolegnia* the zoospores escape from the sporangium by a terminal papilla, the spores swim away separately, encyst, and swarm a second time before germinating. Sporangia are renewed by internal proliferation. In *Achlya* the zoospores encyst at the mouth of the sporangium, forming a hollow sphere; after a few hours the spores escape from their cysts and swim away. New sporangia are formed by lateral branches below the first. In *Aphanomyces* de Bary we find the same type of spore-discharge as in *Achlya*, but the sporangia are thread-like and the zoospores are arranged in a single row. In the genus *Dictyuchus* Leitgeb emend. Couch the spores encyst within the sporangium, emerge separately by individual openings, their empty cysts leaving a „cell net" structure on the inside of the sporangial wall. The genus *Isoachlya* Kauffman has zoospores escaping and swarming separately as in *Saprolegnia*, secondary sporangia are formed by lateral branches as in *Achlya* or by internal proliferation as in *Saprolegnia*.

Many investigators have drawn attention to the fact that the morphological characters of the *Saprolegniaceae* are very variable and easily influenced by environmental conditions.

Coker (1923) in the introduction to his Monograph (p. 4-10) gives a review of „abnormal" discharge of sporangia and behaviour of zoospores in a number of species of the *Saprolegniaceae*, as observed by different authors.

In some species, however, two methods of sporangium discharge are typically present, each of which is characteristic of a different genus. In *Thraustotheca primoachlya* Coker et Couch the sporangia

are at first of the *Achlya* type, from the later formed sporangia, however, the spores are liberated by disintegration of the sporangial wall, as characteristic of the genus *Thraustotheca*.

In *Dictyuchus achlyoides* Coker the initially formed sporangia are also of the *Achlya* type and are followed by those typical for the genus *Dictyuchus*.

In a recent investigation of the factors controlling the formation of these sporangial types SALVIN (1942) proved that some component of the staling material in the culture medium (hemp seed in water) inhibited the formation of the *Achlya* type of sporangia. Thus, after some days of growing, the sporangia of the *Achlya* type decreased in number and only the sporangia typical of the genus were formed.

The genus *Protoachlya* Coker is in its vegetative characters intermediate between the two main genera, as it has sporangia of the *Achlya* type and of the *Saprolegnia* type. LOUNSBURY (1930) proved that this feature of *Protoachlya paradoxa* remains stable under normal and various experimental culture conditions.

M. and Mme. FERNAND MOREAU (1935, p. 355) conclude from a number of observations upon the varying behaviour of sporangia and zoospores „qu'aucun des genres secondaires (such as *Dictyuchus*, *Thraustotheca*) de ces champignons n'est indiscutable, et que les deux genres principaux, *Saprolegnia* et *Achlya*, sont eux-mêmes réunis par des intermédiaires".

Although these variations in the methods of asexual reproduction do not invalidate the distinctions between the genera of the *Saprolegniaceae*, it is important to keep in mind, that the characteristics on which this classification is based do fluctuate and are subject to many irregularities.

Problems may arise in the classification of the genera — still greater difficulties may be encountered in separating some of the species within a genus. A number of species can be clearly distinguished by well defined characteristics (e.g. *Saprolegnia asterophora*, *Achlya oligacantha*), whereas other species are separated only from one another by small differences, which often vary, so that the species merge into one another and sometimes completely overlap. These complex species seem to consist of a number of closely related forms and it is only a matter of personal conviction how far one shall go in separating these form-groups into species, variaties or strains. One instance studied by several investigators may illustrate this fact.

Saprolegnia ferax (Gruith.) Thuret has usually been described with antheridia on less than 10 % of the oogonia, *Saprolegnia mixta* de Bary has antheridia on about 50 % of the oogonia, whereas in *Saprolegnia monoica* Pringsheim all the oogonia are provided with antheridia. COKER, cultivating a strain of *S. ferax*, was able to obtain from 1 to 95 % of oogonia-bearing antheridia, depending on the medium used. KLEBS (1899) observed that in *S. mixta* the number of antheridia varied from 0 to 90 %. PIETERS (1915) grew *S. monoica* in 0.05 % haemoglobin solution, he observed antheridia on 0 to 17 % of the oogonia, the species normally having all oogonia furnished with antheridia. FORBES (1934) suggests that the three species mentioned might be regarded as different growth forms of one species. The same may hold true of other forms of the ferax group described as separate species, such as *S. esocina* Maurizio, *S. bodanica* Maurizio, *S. lapponica* Gäumann, and probably several other species.

COKER divides the genus *Saprolegnia* in five species-groups. Within the two main groups, the diclina group and the ferax group, he maintains a number of species, although these, as M. et Mme. FERNAND MOREAU (1939, p. 242) well express it, are often only separable by ,,des caractères de plus ou de moins''.

In the course of the present investigation a considerable number of strains belonging to the ferax and the diclina group were collected, with the intention of studying to what extent single spore cultures in prolonged cultivation maintain the same features as their parent cultures. During the last year of the war, the total lack of gas and electricity and the great scarcity of agar, caused us to concentrate entirely on the maintenance of the culture collection of the C.B.S., and this material could not be kept.

III. SPECIES OBSERVED.

The following species have been recorded in Holland before:

By OUDEMANS 1904: *Achlya prolifera* (Nees) de Bary.

By BOEDIJN 1921: *Sapromyces Reinschii* (Reinsch) Fritsch, *Saprolegnia monoica* Pringsheim, *S. ferax* (Gruith.) Thuret and 1923: *S. diclina* Humphrey, *S. mixta* de Bary, *Aphanomyces laevis* de Bary, *A. helicoides* de Bary, *Rhipidium europeum* (Cornu) v. Minden, *Blastocladia Pringsheimii* Reinsch.

In the present record the classification used by FITZPATRICK (1930) has been followed. Some of the less common species are

described in more detail and observations made on cultures are noted. When necessary, to explain the terms used, a short general description of the genus, adapted from other authors (COKER, FITZPATRICK, KANOUSE), precedes the observations.

ORDER CHYTRIDIALES

Genus *Olpidiopsis* (Cornu) Fischer 1892.

Species of this genus are parasitic in water moulds. Mycelium lacking, thallus intramatrical, sporangium smooth-walled, usually oval, one or more exit tubes penetrate the wall of the host. Swarmspores biciliate. Wall of the resting spore (oogonium) rough or spiny, companioncell (antheridium) present.

Olpidiopsis saprolegniae (Cornu) emend. Fischer. RABENHORST's Krypt. Fl. I, part 4, p. 38, fig. 4, 1892.

Localities: Ommen, April 1944 on *Achlya colorata*. Botanical gardens Baarn, July 1943, parasitizing *Saprolegnia diclina*.

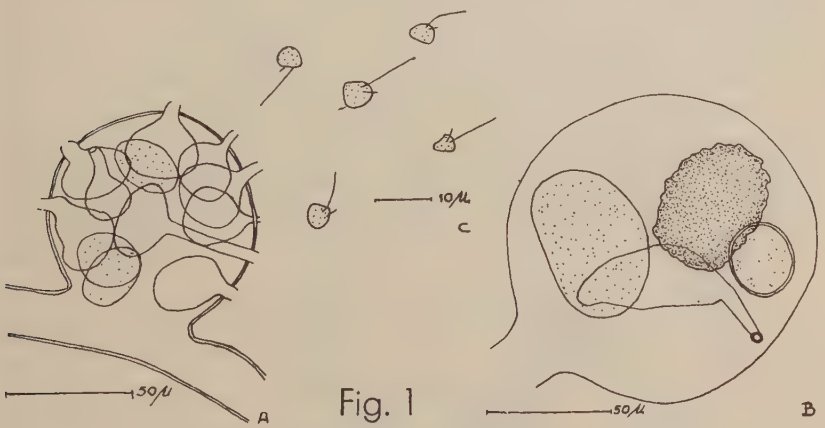


Fig. 1

Olpidiopsis saprolegniae.

a. Sporangia, with exit tubes.
b. Two sporangia (one empty) and young oogonium with antheridium, inside an oogonium of *Saprolegnia diclina*.
c. Zoospores, stained with iodine.

This *Olpidiopsis* was cultivated with the *Saprolegnia* until June 1944. The size of the oogonia (50 × 39 µ) was somewhat smaller than that given by FISCHER (78 × 65 µ). The diameter of the antheridia (20 — 26 µ) lies in between the measurements given by

FISCHER (28 — 30 μ) and COKER (18 μ). Zoospores stained with iodine showed one short and one long cilium, attached at a small distance from each other (fig. 1, c). COKER (1923, plate 62, figs. 9 and 10) draws two cilia of equal length arising from the same spot; in some spores (fig. 10) attached to the anterior end of the spore, in others (fig. 9) laterally inserted. According to FISCHER (1892, p. 38) the spore has two cilia „one at the pointed apex, the other usually longer, attached laterally".

ORDER BLASTOCLADIALES

Genus *Gonapodya* Fischer 1892.

The hyphae of the filamentous mycelium are segmented by pseudosepta, usually accompanied by constrictions. Sporangia terminal, proliferating. Zoospores mostly with one cilium. Sexual reproduction not definitely known.

Since sexual organs are not known with certainty the genus is rather difficult to place.

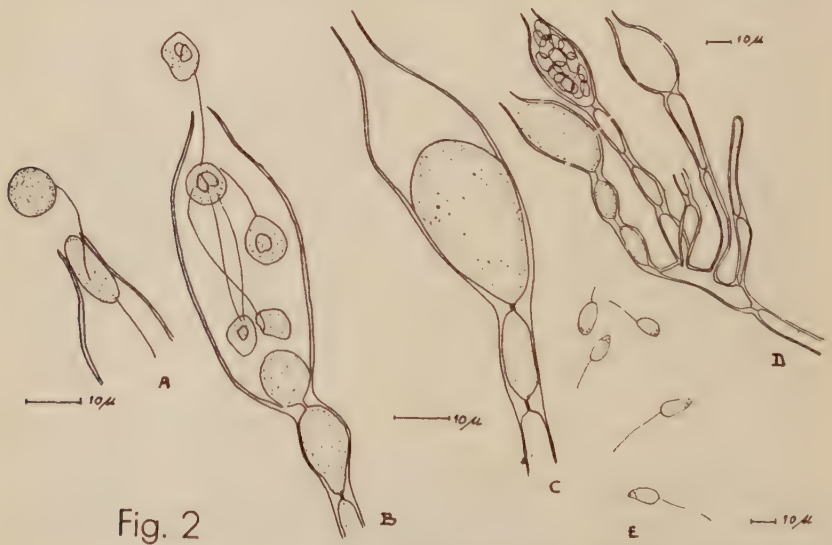


Fig. 2

Gonapodia polymorpha.

a. Sporangium-mouth discharging spores.
b. Sporangium with zoospores, stained with iodine.
c. Proliferating sporangium.
d. Part of thallus showing a proliferating sporangium and a sporangium freeing its spores.
e. Uniciliate swarmspores.

The genus *Gonapodya* has two species which are sometimes difficult to distinguish. Intermediate forms seem to connect the two types (PETERSEN 1910 p. 533). The differences are: *G. prolifera* (Cornu) Fischer hyphae more or less regularly segmentated by pseudosepta. Sporangia elongate (130—250) × (22 — 30) μ. *G. polymorpha* Thaxter: segmentation often ill-defined. Sporangia oval (20 — 60) × (12 — 30) μ.

Gonapodya polymorpha Thaxter. Bot. Gaz. **20**, p. 481, Pl. 31, figs. 11—16, 1895.

Locality: Botanical gardens, Baarn, Nov. 1943, found on an apple lying in water.

The sporangia of this specimen measured (50—118) × (18—23) μ. As the length of most of the sporangia was not more than 64 μ this specimen is within the limits of *G. polymorpha*.

The fungus was in active condition and several sporangia were observed while discharging their spores. The spores leave one by one through the narrow exit pore, round up when free and swim slowly away (fig. 2, a). Zoospores in a sporangium stained with iodine at the moment it was emptying, demonstrated one long cilium at the distal end (fig. 2,b). Sporangia of greater length (109—118 μ) were measured after the apple with the fungus had been lying in tap water (frequently renewed) for several weeks. It would be interesting to know, to what extent, through change of environmental conditions *G. polymorpha* could be induced to assume the characteristics ascribed to *G. prolifera*. My material was too scanty to allow such experiments.

ORDER SAPROLEGNIALES

Family Saprolegniaceae.

Genus *Saprolegnia* Nees v. Esenbeck 1823.

F e r a x-G r o u p :

Several species of the ferax group are common in Holland and have been collected many times from different localities throughout the year.

As this material has not been properly worked over for the reasons given above, only those forms that could be easily placed in one of the described species, will be mentioned here.

Saprolegnia ferax (Gruith.) Thuret. Ann. Sci. Nat. Bot. Series 3, **14**, p. 229, pl. 22, 1850.

Saprolegnia mixta de Bary. Bot. Zeit. **41**, p. 56, 1883.

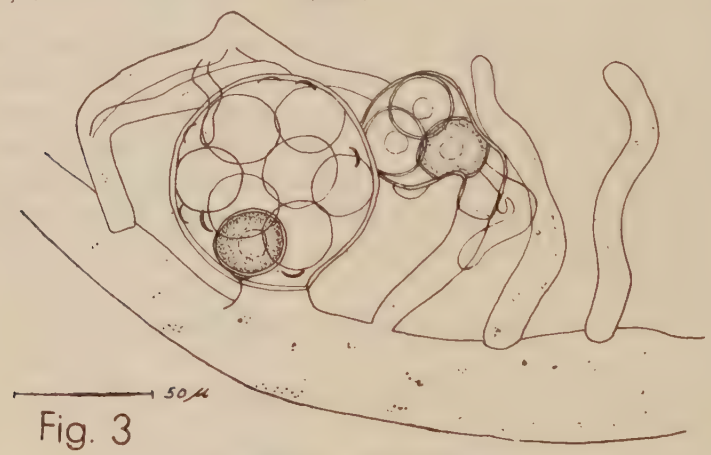
Saprolegnia monoica Pringsheim. Jahrb. f. wiss. Bot. **1**, p. 292, pl. 19, 20, 1858.

Saprolegnia monoica var. *glomerata* Tiesenhausen. Arch. Hydrobiol. Plankt. **VII**, p. 277, figs. 6—8, 1912.

For synonyms in this group see COKER (1923).

Occasionally a fungus was isolated with antheridia containing oospores. MAURIZIO (1899) described this peculiarity for his species *Saprolegnia paradoxa* (not *S. paradoxa* Petersen). BROWN (1938) reports *Saprolegnia paradoxa* Maurizio in Great-Britain, but she has not seen antheridia, so why this specimen is assigned to *S. paradoxa* is not quite clear. NEWBY (1948, b) isolated this fungus from a dead Leuciscus collected at Haslemere Surrey.

NEWBY made an attempt to stimulate the production of these egg-containing structures by growing his isolate in synthetic solutions, but no result was obtained.



„Saprolegnia paradoxa" Maurizio.

Fig. 3 shows an oospore-bearing antheridium observed on a strain isolated from twigs collected from the pond at Eerde, Ommen, April 1944. The oogonia of this isolate measured 49—75 μ, oospores 6—20 in an oogonium, 23—26 μ thick.

On the egg-containing antheridium there was also an antheridial branch, whether an antheridium was formed could not be seen, as the tip of the branch was covered by the „paradoxical" anther-

idium. It was observed on several strains that these peculiar egg-containing structures became less in number after several transfers to fresh hemp seeds. After some time the isolate could not be distinguished from an ordinary *Saprolegnia monoica* var. *glomerata*.

Diclina Group:

Forms of this group were collected from at least ten different localities. One strain was isolated from diseased *Xenopus larvae*, obtained from a Zoological laboratory at Utrecht.

Saprolegnia anisospora de Bary has been transferred to the genus *Isoachlya* by COKER (1937).

Asterophora Group:

Saprolegnia asterophora de Bary. Jahrb. f. wiss. Bot. **2**, p. 189, pl. 20, figs. 25—27, 1860.

Locality: Pond at Eerde, Ommen, Febr. 1944. Temperature of the water about freezing point. Twigs collected from the pond were put in glass dishes with sterile tap water, provided with hemp seeds. After a few days cultures of *S. asterophora* developed in several of the dishes.

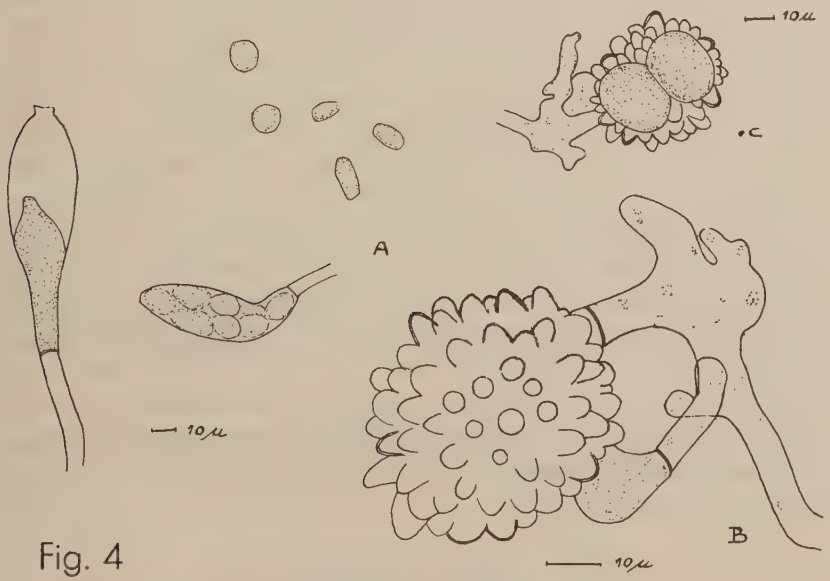


Fig. 4

Saprolegnia asterophora.

a. Proliferating sporangium, sporangium about to discharge its spores, zoospores (cilia not shown).
b. Oogonium with antheridium (focused on the oogonial wall).
c. Oogonium with two oospores.

This fungus is rather rare, although it has a wide distribution. It has been reported in America by HUMPHREY (1892, seen only from preparations by TRELEASE), by PIETERS (1915) and by COKER (1923) who found it only three times in the more than two thousand collections which he made in Chapel Hill, twice in Febr. 1918, once Febr. 1922. In Europe it has been reported by DE BARY (1860), FISCHER (1892), ISTVANFFI (1895), VON MINDEN (1912), APINIS (Latvia, April 1928, found in moist soil of a meadow, and August 1929), HÄRYÉN (Finland 1928 on a fish), PETERSEN (1910) collected it twice in Denmark, once Jan. 1907 from a dead Leuciscus, he did not see any zoosporangia, LUND (1934) found the species in two localities, March and April 1931.

Notes about cultures of the strain collected at Eerde: On oatmeal, cornmeal and other agar media growth good, hyphae very varying in width, sometimes clavate or bag-shaped. Sporangia lacking, oogonia not observed. Cultures transferred from agar media to hemp seed in water developed sporangia and oogonia. The best development was obtained if care was taken that little or no nutrient medium was transferred with the mycelium. This confirms the observation of PIETERS (cited by COKER 1923, p. 65) that this species „thrives on extremely small quantities of food in solution".

Sporangia are scarce, they contain mostly but few spores, which after being discharged swim slowly away. The zoospores are rather large, upto 16 × 10 μ at the moment they leave the sporangium, 11 μ when encysted. The sporangia proliferate from within. The oogonia are densely covered with short, blunt papillae. Oogonia, including papillae, about 44 μ thick. One or two oospores in an oogonium. The oogonial stalk has many side branches, only one or two (sometimes none) of these develop into antheridia (Fig. 4,b and c).

The species was new to the collection of the C.B.S. This strain has been in culture mostly on cornmeal or oatmeal since 1944. In 1948 transfers from agar media to hemp seed in water again developed oogonia.

Genus *Isoachlya* Kauffman 1921.

Isoachlya monilifera (de Bary) Kauffman. Am. Jour. Bot. **8**, p. 231, 1921.

Saprolegnia monilifera de Bary. Bot. Zeit. **46**, p. 692, 1888.

Localities: collected twice from the pond at Eerde, Ommen, February 1943 and February 1944. Once isolated from soil near a pool in a wood at Baarn.

Isoachlya anisospora (de Bary) Coker. COKER and MATTHEWS, North American Flora **2**, part 1, p. 26, 1937.

Saprolegnia anisospora de Bary. Bot. Zeit. **46**, p. 619, 1888.

Localities: Pond at Eerde, Ommen, February 1944. Pond in botanical Gardens, Baarn, January 1944.

As the name indicates the zoospores are of unequal size, large (13—14, upto 17 or even 20 μ) and small (8—9 μ), also intermediate sizes occur, a single sporangium usually contains spores of only one size.

There has been a controversy concerning the structure of the oospore in this species. In his diagnosis DE BARY (1888) described eccentric eggs. COKER (1923) thought that DE BARY's observation was erroneous and that his figures show eggs that had been breaking down. In 1937, however, COKER corrects his former statement and agrees with DE BARY that the eggs have an eccentric structure. SAKSENA and BHARGAVA (1944) describe an *Isoachlya anisospora* var. *indica*, which differs from the main species in the structure of the oospore which is either centric or subcentric, but never eccentric. BHARGAVA (1946) studied the development and cytology of the sexual organs of *I. anisospora* var. *indica* and definitely established the occurrence of fertilization in this variety.

On account of the sporangial characters, COKER and MATTHEWS (1937) transferred this species from the genus *Saprolegnia* to *Isoachlya*, a change already suggested by APINIS (1929). In the latter genus the species *Isoachlya eccentrica* Coker et Matthews also has eccentric oospores, while all *Saprolegnia* species have centric oospores. *Isoachlya anisospora* is not very common. It has been found in North America, Japan, Germany, Latvia, Denmark and quite recently NEWBY (1948, a) recorded the species in Great-Britain.

Genus *Protoachlya* Coker 1923.

Protoachlya paradoxa (Coker) Coker. The Saprolegniaceae, p. 91, pl. 26—28, 1923.

Achlya paradoxa Coker. Mycologia, **6**, p. 285, 1914.

Isoachlya paradoxa Kauffman. Am. Jour. Bot., **8**, p, 231, 1921.
Locality: This species was collected from a shaded pool in a wood near Arnhem, July 1944.

Protoachlya paradoxa is a comparatively rare species. COKER (1923) found it 35 times in the several thousand collections he made around and in Chapel Hill (North Carolina). LOUNSBURY (1930) collected it once in several hundred collections made near Madison (Wisconsin), summer 1927. APINIS reports the species from Latvia. He found it once, north of Riga, July 1929 and cultivated it only for a short time. CHAUDHURI and KOCHHAR (1935) collected this fungus twice in India (Lahore district of Punjab), no details are mentioned concerning the season.

This species is interesting not only from the point of view of its distribution and rarity, but also because it combines several of the characteristics we find in the different genera of the *Saprolegniaceae*. COKER (1914) after first describing it as an *Achlya* created a new

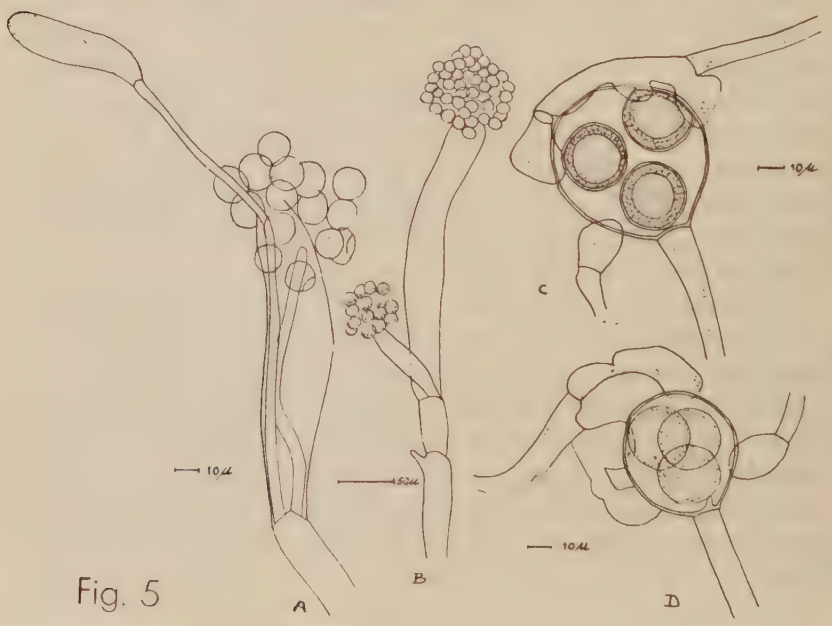


Fig. 5

Protoachlya paradoxa.

a. Sporangium with cluster of empty spore-cysts. New sporangium formed by internal proliferation.
b. Sporangia, the lateral branch has just discharged its spores.
c. Oogonium with mature oospores, showing centric and subcentric structure.
d. Oogonium with diclinous antheridia.

genus, *Protoachlya*, for this fungus (1923). He does not agree with KAUFFMAN who transferred it to *Isoachlya*.

Since 1944 *Protoachlya paradoxa* is in the collection of the C.B.S.

Cultural characteristics.

The vegetative growth on oatmeal agar is like that of an *Isoachlya*. Within a week the tube is filled with aerial mycelium, the growth is much denser than that of *Saprolegnia* species on the same medium. The mycelium of *Protoachlya paradoxa* does not contract the agar, as *Achlya* species do, when the culture gets older. On hemp seed in water the growth is delicate, the hyphae are slender. The stout, bristle-like hyphae, typical of *Achlya* are entirely lacking. Sporangia abundant. Secondary sporangia are usually formed by cymose branching, occasionally, however, a hypha grows from the bottom wall of an empty sporangium, forming a new sporangium outside the old (fig. 5, a). Thus this species, in forming its sporangia, combines both the *Achlya* type (cymose branching) and the *Saprolegnia* type (proliferation) with this difference that the secondary sporangia formed by proliferation always develop entirely outside the old sporangium.

Some notes on the behaviour of the spores on emerging will now be given. These notes were made at the moment of observation (29-9-1944).

1) Sporangium rather small. The sporangium empties slowly. No movement of the spores inside the sporangium is seen. Two spores are very slowly driven out. The cilia, trailing behind, are clearly visible. All spores remain at the mouth of the sporangium. After a slight rocking movement the spores become still.

2) Large sporangium: The spores in the top of the sporangium are moving gently, in the gaps between the spores the lashing of the cilia can be seen. The spores charge out with great rapidity at first, then the rate of discharge slows down and the rest of the spores leave the sporangium sluggishly. The spores remain in a mass at the sporangium mouth, showing a rocking movement. Several spores move actively past their neighbours and push between the other spores into the spore cluster again. After ten minutes all rocking movement comes to an end.

3) Sporangium emptying, all spores swim slowly away.

Gemmae abundant, spherical to pyriform, 40—60 μ.

Oogonia: Some months after the fungus had been isolated oogonia

were seen in a culture transferred from oatmeal agar to hemp seed in water. The oogonia developed in a dense weft of mycelium close to the hemp seed, usually on lateral branches. The oogonia are spherical, 42—75 μ in diameter, the oogonial wall is 2 μ thick. Pits were seen where the antheridia touch. (On the larger oogonia, COKER saw free pits also). There is a small upgrowth from the separation wall into the oogonium.

Oospores: centric or subcentric, 2—10 (usually 4—5) oospores in one oogonium. Diameter of the eggs 24—30 μ (averaging about 27 μ), fig. 5, c. Antheridia numerous, several to one oogonium. Antheridial branches slender, diclinous. Antheridia large, almost pouch-like, sometimes branched, touching the oogonia with their tips. Fertilization tubes present. The young antheridia are densely filled with protoplasm, the wall of the empty antheridia is refractive, fig. 5, d.

Striking features: The comparatively small oogonia, rather regular in size with the big pouch like antheridia, remaining visible long after their contents are discharged.

The formation of oogonia in this fungus is rather rare and has been the subject of many experiments.

CHAUDHURI and KOCHHAR (1935) obtained oogonia on house fly in 0.1 % solution of leucine. LOUNSBURY in his „Investigations on the Nature of *Protoachlya paradoxa*" (1930) cultivated the fungus on a great number of different media, such as pea extract, spinach broth, milk, leucine solution, haemoglobine solution, various mineral salts in different concentrations, different pH and temperatures and light of different colours. None of these circumstances, however, would induce the fungus to form oogonia. Three times in these experiments, covering a period of two years, he obtained oogonia, but no direct correlation between the experiment and the result could be established. Eventually LOUNSBURY succeeded in securing the formation of oogonia when he cultivated the fungus on hemp seed in tubes, in such a way that the fungus was covered by a depth of 4—7 (or more) cm of water. From these experiments LOUNSBURY assumes that the appearance of sexual organs bears some relation to free oxygen. His method was tried by the writer, but without any success.

COKER (1923) also describes a number of experiments made to induce the formation of oogonia. But, as COKER expresses it, „the appearance of the oogonia seems to be entirely whimsical at times".

And indeed „whimsical" seems the best term to describe the occurrence of sexual reproduction in this fungus.

The rarity of oogonia might explain why this fungus has been recorded so seldom, as only sexual organs make identification of the species possible in this group. But on the other hand only a superficial observer would mistake this species for a sterile *Achlya*. The behaviour of the spores on emerging and the two types of sporangia which appear under nearly all circumstances (LOUNSBURY) characterize this species even in its vegetative state.

Genus *Achlya* Nees v. Esenbeck 1823.

Achlya colorata Pringsheim. Sitzungsber. der Akad. der Wissensch. Berlin, p. 855, pl. 14, figs. 12, 15—31. 1882.

Achlya racemosa var. *stelligera* Cornu. Ann. Sci. Nat., **15**, p. 22, 1872.

A common species in Holland. Collected from many different localities, it seems to be especially frequent during the winter months.

Achlya racemosa Hildebrand. Jahrb. f. Wiss. Bot., **6**, p. 249, pl. 15, 1867.

This fungus was collected several times from the pond in the botanical gardens, Baarn. ZIEGLER (1948) studied the germination processes of the oospores in this species and in several other species of the *Saprolegniaceae*.

Achlya radiosa Maurizio. Mitt. d. Deutsch. Fischerei-Vereins, **7**, Heft 1, p. 57, figs. 18 and 19, 1899.

Achlya decorata Petersen. Bot. Tidskr., **29**, p. 383, fig. III, a and e, 1909.
Achlya asterophora v. Minden. Krypt. Fl. Mark Brandenb., **5**, p. 549, fig. 2c, 1915.

Locality: a marshy place in a brook near Houthem, South Limburg, April 1947.

Achlya radiosa has not been found in America. OBEL (1910) employed this species, under the name *Achlya decorata* Petersen, in his experiments on the formation of oogonia in *Achlya*.

Achlya oligacantha de Bary. Bot. Zeit. **46**, p. 647, pl. 10, fig. 1, 1888.

Locality: Pond at Eerde, Ommen April 1944. The water-sample was taken right from the surface of the pond.

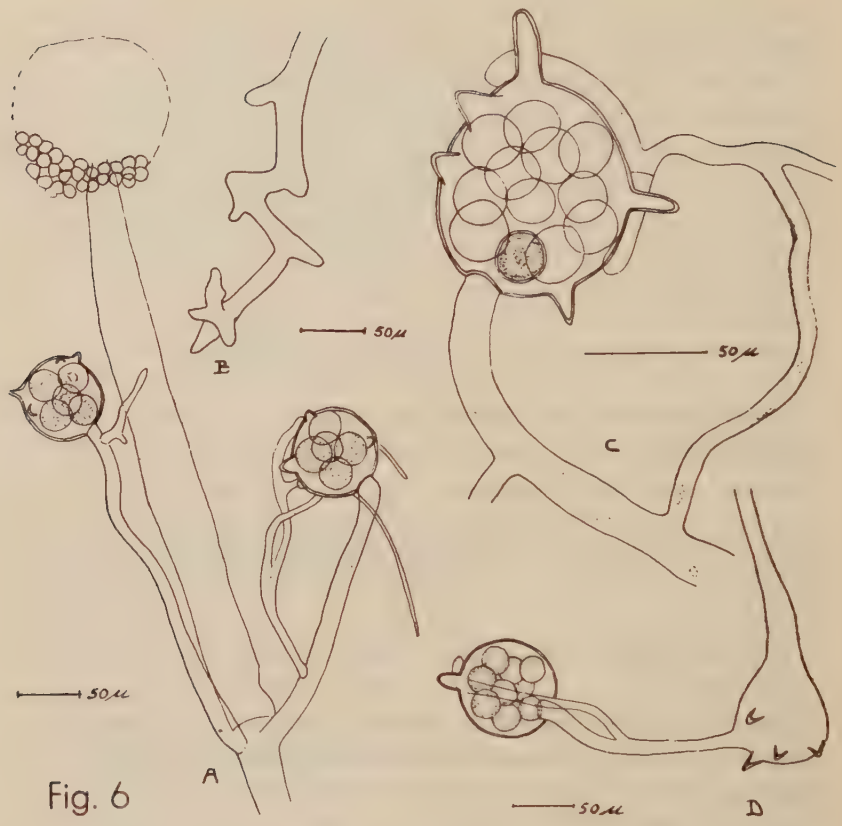


Fig. 6

Achlya oligacantha.

a. Sporangium and oogonia, one with androgynous antheridial branches.
b. Tip of hypha, developed on the surface of the water.
c. Oogonium with androgynous antheridium.
d. An oogonial initial, already decorated with spines, halted in its development; a complete oogonium developed from a branch of this oogonial initial.

This fungus has been reported only 4 times. De Bary found it in water from a puddle at Kork (Baden, Germany) in June 1881. He cultivated it pure until 1883. In Denmark it has been recorded by Petersen (1910) and by Lund (1934), who found it in May 1932 on twigs, pH of the water 7.3. Both authors observed it only once. Some years ago Haines, a pupil of Dr Ivimey Cook, collected this species near Cardiff in November [1]).

[1]) Dr Ivimey Cook kindly allowed me to examine the unpublished notes.

As there are only two rather short descriptions of this species and hardly any illustrations, some observations made upon cultures will be given here.

Cultures on hemp seed in water:

Growth not very dense but rather extensive, reaching a diameter of about two centimetres in ten days. Mycelium slender, sometimes, near the surface of the water, some rather thick hypae develop (15—30 μ) terminating in broad antler like ramifications (fig. 6, b). Sporangia not frequent. In ordinary tap water (poor in mineral salts in this district) they do not develop at all. In a culture transferred from tap water to sterilized pond water seven sporangia were counted. They emptied normally for the genus. Often the spores germinate in the spore sphere at the sporangium mouth. A vigorously growing culture from oatmeal agar transferred to hemp seed in water, developed oogonia after four days, oospores were visible on the seventh day. The oogonia develop terminally on rather slender hyphae near the edge of the culture, some are scattered all over the mycelium. The size of oogonia and oospores of this strain agrees with the measurements given by Lund, only the oospores are somewhat smaller. Oogonia (without projections) 70—106 μ, wall unpitted, papillae 0—8 on an oogonium, 4—20 μ long, blunt. Oospores 6—14, mostly 7—10 in an oogonium, 21—26μ, extremes of 17—30 μ are found.

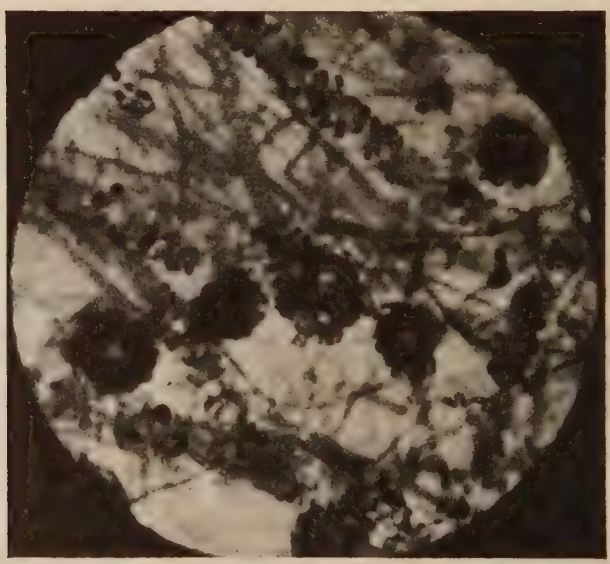
De Bary in the only figure he gives of this fungus shows an oogonium with oospores of unequal size, one of them squeezed into an outgrowth. The contents of the oospores are homogenous when young, there is a tendency to an early degeneration of the oospores, after a week their contents may break up into several large oil-drops.

Not infrequently an oogonial initial already decorated with projections, but lacking antheridia, does not finish its development. It proliferates to form a second oogonium, in which, if provided with antheridia, the oospores mature (fig. 6, d). In this species fertilization seems to be of importance for the development of the oospores. A similar proliferation of oogonial initials was observed on *Achlya ambisexualis* by Raper (1939).

As to the antheridia, I can confirm Lund's observation that the androgynous antheridial branches are more numerous than the diclinous. From twenty-one oogonia sixteen had androgynous



a



b

Fig. 7. *Achlya bisexualis.*

a. Numerous antheridial branches coiled around the oogonial stalks and oogonial initials of the female strain. Mounted in lactic acid with fuchsine. × 60.

b. Oogonia with oospores. Mounted in lactic acid with cotton blue. × 193.

The microphotographs were taken thirty-six hours after the male and female cultures on hemp seed were placed together in a Petri dish.

antheridia, five had diclinous, two of these had androgynous antheridia as well. Antheridial branches slender, antheridia relatively small, elongated, applied with their sides against the oogonia.

Achlya oligacantha grows well on cornmeal and oatmeal agar, but no oogonia were observed on these media. After four years of cultivation on these media the strain, however, readily develops fructification organs when transferred to hemp seed in water.

Achlya bisexualis Coker et Couch. COKER, Jour. Elisha Mitchell Sci. Soc. **42**, p. 207, pl. 27, 1927.

Locality: a female strain was collected from a stone water-basin containing tropical plants in a hot-house of the botanical gardens at Baarn.

During the course of this investigation several sterile strains of *Achlya* have been isolated from different localities in the surroundings of Baarn. Most strains had chains of elongated gemmae as typical of *Achlya*. One strain, however, was different from the others in having many spherical to ovate gemmae. In many respects this strain agreed with the ,,*Achlya* species? Form without oogonia'' described by COKER (1923 p. 137) and with the ,,*Achlya* lacking sexual reproduction'' which was described by WESTON (1917).

Crossing experiments were carried out with this plant and four other sterile strains of *Achlya*, but no sexual organs were formed. The fungus collection of the C.B.S. contained an *Achlya* species which was sent to this laboratory for identification by Professor MELIN who had collected it in Sweden in 1942. As the specimen never developed sexual organs, it had not been possible to determine the species. This plant I also employed in my crossing experiments. Hemp seed cultures of the Swedish strain were placed in Petri dishes together with the isolate from Baarn. Within twenty-four hours a dense weft of thin hyphae is developing on the line of contact of the two cultures. The slender hyphae originating from the vegetative mycelium of the Swedish plant prove to be antheridial branches. These antheridial branches entwine the oogonial initials and even the oogonial stalks of the female plant (the Baarn isolate) to such an extent that they may cover their entire surface. In one case oospores were observed already thirty-six hours after the two strains were mated (fig. 7, a–b). The oospores rarely mature, very soon their contents break up into large oil-drops. The same observation was made by COKER (1927) on his strain of *Achlya bisexualis*.

The oogonia are borne either terminally or on lateral branches, 46—91 μ thick, oospores 5—10 per oogonium, 20—28 μ in diameter. The resistant spores of the female strain are borne singly, very rarely in a short chain. In this respect this strain differs from the specimen described by WESTON (1917) in which the resistant spores were formed in chains.

Genus *Dictyuchus* Leitgeb 1869, emend. Couch 1931.

Dictyuchus species.

Two sterile strains of *Dictyuchus* were collected, one from an aquarium with goldfish, the other from a submerged medlar, botanical gardens, Baarn, September 1945. Both strains resembled the plant described by COKER (1923, p. 151) and were almost identical with a *Dictyuchus* strain of IVIMEY COOK, kept in the collection of the C.B.S. Since COUCH (1924, 1926) discovered heterothallism in this genus, we know that the non-sexual forms may represent male or female strains of a heterothallic *Dictyuchus* species.

Genus *Aphanomyces* de Bary 1860.

Aphanomyces laevis de Bary. Jahrb. f. wiss. Bot. **2**, p. 179, pl. 20, figs. 17, 18, 1860.

This species was collected several times from a water basin in a hot-house of the botanical gardens and from other localities in the surroundings of Baarn. *Aphanomyces helicoides* v. Minden was recorded in Holland by BOEDIJN (1923). According to COKER (1923, p. 168) this species cannot be separated from *Aphanomyces laevis*.

Family Leptomitaceae

Genus *Apodachlya* Pringsheim 1883.

Hyphae constricted into segments by pseudosepta. No rhizoids. Zoosporangia terminal, oval to pyriform, broader than the hyphal segments. Oogonia monosporous.

Apodachlya brachynema (Hildebrand) Pringsheim. Ber. d. Deutsch. Bot. Gesell. **1**, p. 289, 1883.

Leptomitus brachynema Hildebrand. Jahrb. f. wiss. Bot., **6**, p. 261, 1867.
Apodya brachynema (Hildebr.) Cornu. Ann. Sci. Nat., Series 5, **15**, p. 14, 1872.

Locality: moat surrounding Castle Eerde near Ommen, Febr. 1943.

This fungus was cultivated for a short time on ant-pupae and hemp seed. As this species was already present in the collection of the C.B.S. no attempt was made to obtain pure cultures of this specimen.

For a detailed description see COKER (1923, p. 173—176) and KANOUSE (1927, p. 338—339). COKER interprets the so called „resting spores" as oogonia. As to COKER's supposition that the suboogonial cell might be considered as an antheridium, KANOUSE is of the opinion that this statement wants further proof, based on cytological investigation.

Genus *Rhipidium* Cornu 1871.

Rhipidium americanum Thaxter. Bot. Gaz., **21**, p. 320, 1896.

Locality: found on a decaying submerged medlar, collected from the pond at the botanical gardens, Baarn, October 1943.

The thallus of the plant is attached to the substratum by means of rhizoids. The distal portion of the thallus is variously lobed or branched. The lobes give rise to slender cylindrical branches, bearing the sporangia and sexual organs. These branches are constricted at their point of origin and before delimitating the reproductive cells. Sporangia are borne singly at the ends of the branches, secondary sporangia develop on side branches arising immediately below the subsporangial constriction; sporangia ovoid $(54 — 64) \times (37 — 45)\ \mu$.

The medlar with the fungus was kept for two weeks in tap water, renewed by constant dripping from the tap. It was then placed in sterile water. After forty-eight hours zoospores were observed. The zoospores, when emerging from the sporangium, are kept together in a vesicle, forming a short column. Soon the thin membrane ruptures and the zoospores swim away one by one. From one sporangium not all zoospores had emerged. The movement of particles inside the sporangium, caused by the lashing of the cilia of the remaining zoospores, was observed, although no cilia were seen. The protoplasm of the zoospores is coarsely granular. Diameter 10—12 μ. After a few hours in water the spores germinate by germtubes (fig. 8, d).

Oogonia are borne terminally on narrow branches which are constricted immediately below the oogonium. Oogonia spherical,



Fig. 8

Rhipidium americanum.

a. Part of thallus.
b. Sporangia.
c. Sporangium retaining five spores.
d. Germinating zoospores.
e. Oogonium with antheridium showing an early stage in maturation of the single oospore.

diameter about 50 μ. Oospore solitary, globose, thick-walled, with an irregular surface.

Antheridia androgynous. A slender antheridial branch arises from the oogonial stalk immediately below the oogonium. Antheridium 15 μ, applied close to the base of the oogonium (fig. 8, e).

Attempst were made to grow the fungus in pure culture, as VON MINDEN (1916) did with *Rhipidium europeum*. Different sterilized seeds and fruits were tried, but no growth was obtained. A transfer of part of a fungus tuft to a *Chaenomeles japonica* fruit survived for a fortnight, but no further development was seen.

ORDER PERENOSPORALES

Genus *Pythium* Pringsheim 1858.

Pythium undulatum Petersen. An. Myc., 8, p. 531, fig. VIII a and d, fig. IX, 1910.

Locality: this fungus was isolated from submerged plants collected from the pond at the botanical gardens, Baarn, March 1944.

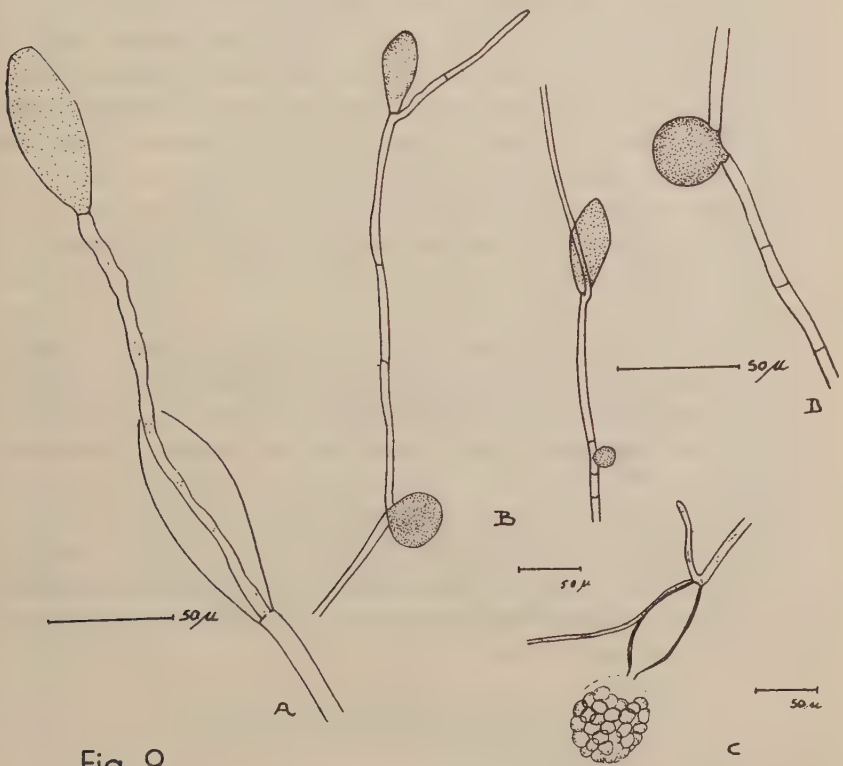


Fig. 9

Pythium undulatum.

a. Proliferating sporangium.
b. Sporangia and young chlamydospores.
c. Sporangium with spore-vesicle.
d. Chlamydospore as typical of this species.

The size of the sporangia of this species seems rather variable: 130 × 50 μ according to PETERSEN, (40 — 45) × (12 — 15) μ SPARROW (1932); (52 — 90) × (26 — 40) μ in this isolate. DISSMANN (1927) gives the following sizes: 76 × 40 μ in 1 % peptone solution; upto 80 μ long in pond water. No sexual organs have been observed in this species.

According to APINIS (1929) and SPARROW (1932) *Pythium undulatum* Petersen is the same as *Pythiomorpha undulata* Apinis. SPARROW, however, does not agree with APINIS in changing the original binominal. In LUND's opinion (1934, p. 49) the two species are different. In *Pythium undulatum* the zoospores are differentiated in a vesicle, whereas in *Pythiomorpha undulata* the spores are discharged from the sporangium directly through a terminal opening (see figs. 3 and 6 in APINIS 1929, p. 235, plate 4).

In the present isolate the spores escaped from a vesicle (fig. 9, c). Chlamydospores with smooth brown walls were rather frequent (fig. 9, b and d). According to APINIS' diagnosis of *Pythiomorpha undulata*, chlamydospores are lacking in this species. LUND for *Pythiomorpha undulata* and PETERSEN for his *Pythium undulatum* do not mention the occurrence of chlamydospores. DISSMANN (1927) and SPARROW (1932) observed rough, brown chlamydospores, whereas MATTHEWS (1931, plate 18) figures chlamydospores with smooth walls.

In the specimen under consideration the zoospores were differentiated in a vesicle and the occurrence of chlamydospores was noted. These characteristics exlude the present specimen from *Pythiomorpha undulata*, so it was identified as *Pythium undulatum* Petersen.

DISSMANN (1927) observed antagonism between *Pythium proliferum* de Bary and *Pythium undulatum*. No antagonism was observed, however, when the present strain of *Pythium undulatum* was cultivated together with a strain of *Pythium proliferum* from the C.B.S. collection.

Summary.

A study was made of aquatic fungi occurring in natural waters in the Netherlands. The following species were recorded for the first time in this country:

Olpidiopsis saprolegniae (Cornu) emend. Fischer

Gonapodya polymorpha Thaxter
Saprolegnia monoica var. *glomerata* Tiesenhausen
Saprolegnia asterophora de Bary
Isoachlya monilifera (de Bary) Kauffman
Isoachlya anisospora (de Bary) Coker
Protoachlya paradoxa Coker
Achlya colorata Pringsheim
Achlya racemosa Hildebrand
Achlya radiosa Maurizio
Achlya oligacantha de Bary
Achlya bisexualis Coker et Couch (oogonial strain)
Dictyuchus species
Apodachlya brachynema (Hildebrand) Pringsheim
Rhipidium americanum Thaxter
Pythium undulatum Petersen

Observations made on cultures of these fungi were noted. A more detailed description has been given of some of the rarer species.

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COMMENT ON PIJPER'S FILM OF MOVING BACTERIA [1])

by

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Some time ago PIJPER (4, 5) published a paper in which he tried to show, that the flagella of bacteria are not organs of motion, but useless appendages, polysaccharide twirls, artifacts, due to the motility of the bacteria. This rather revolutionary point of view is not new, as in 1879 VAN TIEGHEM was of the same opinion. Since however MIGULA in 1897 clearly showed, that this could not be the case, the flagella were generally considered to be organs of motion.

PIJPER's view is based on observations on moving bacteria in dark-field. With much skill he succeeded in filming these bacteria, making use of the always bright sunlight in South-Africa. PIJPER had the kindness to send this film to the ,,Nederlandsche Vereeniging voor Microbiologie'' (Netherlands Society of Microbiology) and it was demonstrated during one of the meetings of this society. Rather than furnishing claims, that the flagella are useless appendages, for different reasons the film showed that bacteria are actually put to motion by their flagella.

According to PIJPER the bacteria move because their body can make an undulating, gyrating movement, which is the same as the motion of the flagella. To make this clear PIJPER furnished motion pictures of bacteria, which seem to draw along their flagella. If the flagella actually consisted of lifeless slime matter, they would show no motion of their own. In the film the flagella are gyrating at a speed much greater than could ever be caused by the movement of the bacteria or by currents. Even non-moving bacteria have

[1]) For more details see: T. Y. KINGMA BOLTJES, J. of Path. and Bact. 60, 275, 1948.

flagella gyrating very quickly and moreover it is clear, that these are moved to and fro by the bacteria in such a way, that it could not be ascribed to the action of currents. If the flagella were only lifeless slime material they would be wrung off in no time when turning at the speed as shown, unless they were extremely elastic. Even then, however, the motion could not go on for long, because the opposing strain would become too great.

PIJPER's film also showed, that the flagella, seen in the darkfield consist of a number of primary flagella, that are not visible separately.

Already in 1932 NEUMANN showed, that the flagella of bacteria become visible in a viscous medium (f.i. a gum arabic solution), because the primary flagella are united to a ,,Zopf'' or tail. Formerly this point of view was strongly disputed by PIJPER.

Although PIJPER can know, that the flagella may be invisible, still he thinks, that the bacteria are swimming without, whenever he does not see any flagella. Obviously the fact that no flagella are visible is no proof, that they are not actually present. The actual presence of flagella, even if invisible in dark-field, can be shown by the following experiment.

A slide is provided with a drop of tannin or silver ethylene solution and on this is placed a coverglass on which formerly motile bacteria have been fixed. The preparation is immediately examined with dark-field illumination. Initially no flagella will be seen, but after 10 to 15 minutes they will become clearly visible by the action of the mordant or the silver solution. In fig. 3 and 4 pictures are given of preparations of *Salmonella typhi* and *Proteus mirabilis*. It is therefore more reasonable to claim, that the bacteria move by means of their unseen flagella. When studying the motion of bacteria, it should always be kept in mind, that part of the flagella may remain invisible and that these may cause a movement of the bacteria which does not seem to agree with the movement of the tail.

The fact, that the flagella are the actual cause of the motion of the bacteria may be substantiated by the following observation. When a *Proteus* is observed in dark-field, it appears to be quite

Fig. 1. *Proteus mirabilis*, 1000×.
Fig. 2. *Spirillum serpens*, 1000 ×. Moving flagella near arrow.
Fig. 3. *Salmonella typhi* in Peppler mordant, 1000 ×.
Fig. 4. *Proteus mirabilis* in silver ethylene solution, 1000 ×.
Fig. 5. *Proteus mirabilis* in India ink, 1000 ×.

sensitive to light. As soon as it enters the light spot, its motion is retarded and then comes to a close, but the flagella keep on moving for a short time. As soon as the flagella do not move any longer a cuvet with aurantia yellow solution is placed before the light source. After a short time the flagella start moving again and as soon as their motion is quick enough, the bacterium anew starts swimming. By removing the filter the bacterium again is brought to a stop and so on. The flagella always start moving before the bacteria do and thus it must be the flagella, that cause the bacteria to move. Fig. 2 shows a spirillum with its flagellum revolving so rapidly, that it is seen as a loop. The exposure time was $^1/_5$ sec. Such movement can never be caused by currents nor by Brownian movement and proves, that the bacterium can set its flagellum in motion.

An experiment of ØRSKOV (3) also shows, that the flagella make very lively movements of their own. A piece of agar, containing the edge of a colony of swarming *Proteus* is taken from a plate. This piece is covered with a coverslip on which a thin layer of India ink is spread out and examined with the microscope. It is remarkable to see the bacteria drawing a clear path through the India ink film. With a strong objective the ink particles can be seen to be swept to the rear, a feature that can never be the result of the motion of the bacterial bodies. The action can be seen still better when the bacteria have come to a halt. The ink particles remain in lively motion and after some time the flagella have swept a clear field around the bacteria as is shown by fig. 5. This proves the flagella not to be merely lifeless slime threads without any movement of their own. That flagella cannot be accepted as artifacts caused by the movement of the bacteria, is also shown by fig. 1. Here we see the picture of a rapidly swimming *Proteus* with many spirals along the full length of its body. Now we can imagine, that the slime thread at the end of the bacterium may take the shape of the spiral, but this does not apply to the lateral threads in the case of *Proteus*.

The fine picture made by VAN ITERSON (1) with the electron microscope at Delft shows most convincingly that the flagella cannot be slime threads (fig. 6).

So it is clear that PIJPER's point of view, that flagella are inutile appendages, cannot be accepted. It remains to be seen, however, whether the film shows anything of the spiral motion of the bacterial

body. It must be observed that PIJPER's choice of organism is not a happy one for this purpose. The typhoid bacteria have a very short body, which certainly cannot make a spiral motion such as PIJPER claims it to do. Moreover the film shows some bacteria spinning quickly around an axis parallel to that of the microscope, a movement that cannot be realised by a spiral motion of the body.

The film shows also some long bacteria, f.i. *B. subtilis*. Often these long bacteria are more or less bent, and turning round when swimming, they may give the impression of making a spiral movement. If they were actually making an undulating gyrating movement with their bodies, the bend should move on along the body. It is seen, however, to remain in the same place and so there is no evidence for the bacterium making itself a spiral movement.

In this connection some observations I have made on spirilla may be reported on. If a spirillum would move on by a right-handed spiral movement of its body, this would cause the body to turn slowly to the left. However when a spirillum was observed, that was marked with a bacterium sticking to it, it appeared that the body always turned in the same direction as the observed apparent spiral movement and therefore it is clear, that we see nothing else than a rigid spiral turning around its axis.

Often I saw spirilla turning around an axis parallel to that of the tube of the microscope (fig. 7D). This could never occur if the body made a spiral motion.

I had the opportunity to observe a very long spirillum bent double (fig. 7C), which was swimming quietly. As after some time it snapped straight, it could be stated that this was actually a single organism. If this spirillum had made a spiral motion with its body it could never have moved forward, as one half of the body would have opposed the direction of motion of the other.

It could also be observed that spirilla sticking together always remain in the position as indicated in fig. 7A and are never seen to come into the position of fig. 7B, a fact which would happen if their bodies made spiral motions. Besides such a motion would be the best way to get rid one of the other.

So not a single fact pointed to spirilla not having the rigid bodies, that they are generally accepted to have.

Finally the attention has to be drawn to the wellknown fact, that the motile bacteria have H-antigen, which is located in the flagella, according to PIJPER in the slime layer. This would lead

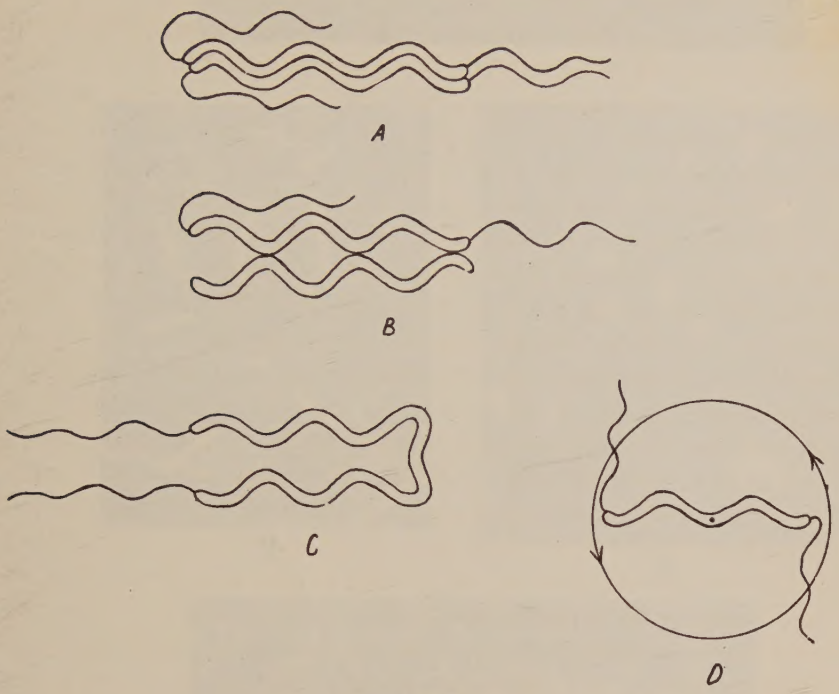


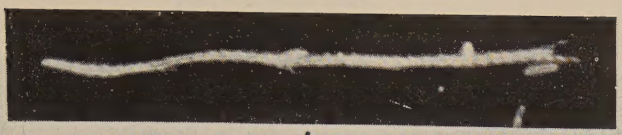
Fig. 7.

to the strange conclusion, that even the bacteria encumbered with a slime layer would be motile and those without non-motile. This argument against PIJPER's view is corroborated by a recent investigation of KAUFFMANN (2), who showed that by addition of antibodies against H-antigen the motility immediately stops because of the agglutination of the flagella. When however O-antibodies were added, the bacteria agglutinized, but the flocks remained motile as the flagella were not disturbed. These experiments also show clearly, that the flagella are organs of motion.

From the foregoing it will be clear, that we still have to consider the flagella as the organs of motion of the rigid bodies of the bacteria.

References.

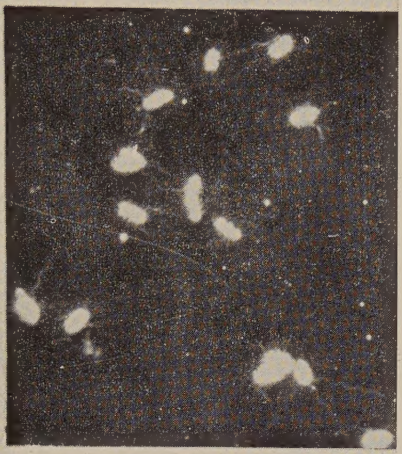
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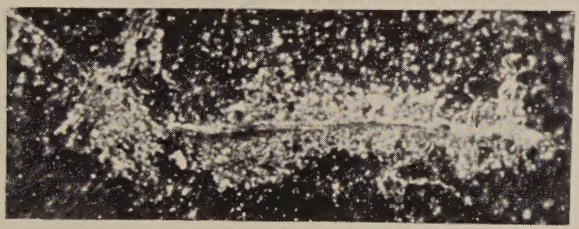
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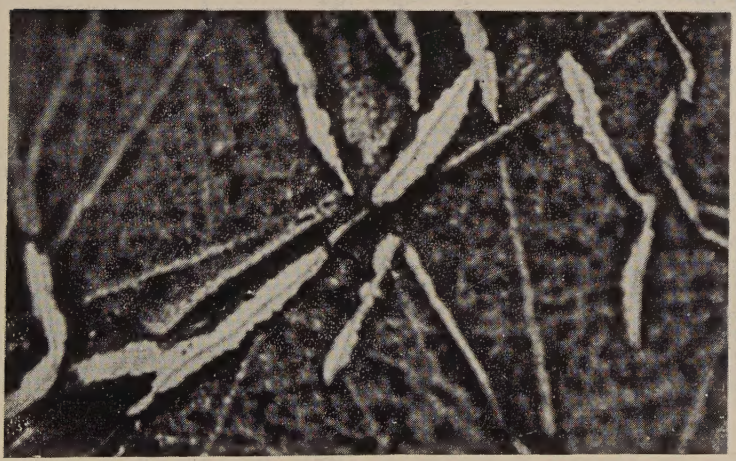
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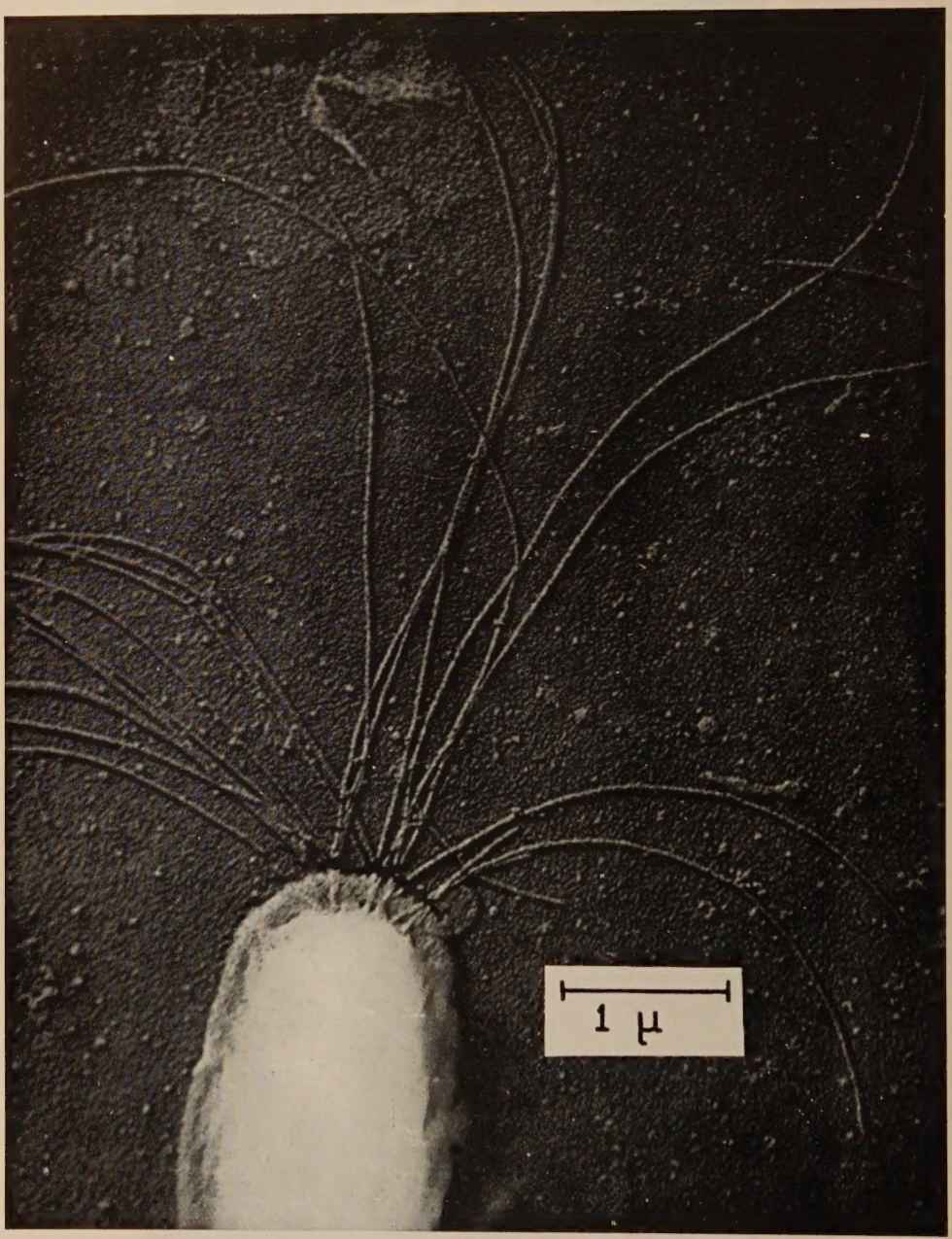


Fig. 6. *Spirillum serpens* by Miss VAN ITERSON, 22500 ×. (Netherlands Institute for Electron Microscopy at Delft). By courtesy Biochimica et Biophysica Acta, Elsevier Publishing Company.